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**Impact of a (poly)phenol-rich extract from the brown algae *Ascophyllum nodosum* on DNA damage and antioxidant activity in an overweight/obese population: a randomised controlled trial**

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Running head: Ascophyllum nodosum (poly)phenols impact DNA damage in the obese.

Abbreviations used: SPE, seaweed (poly)phenol extract; CRP, C-reactive protein; CVD, cardiovascular disease; ROS, reactive oxygen species; TNF- $\alpha$ , tumour necrosis factor alpha; COX, cyclooxygenase; UUREC, University of Ulster Research Ethics Committee; WISP, Weighed Intake Software Program; TF, tissue factor; TOC, Total oxidative capacity; TMB, tetramethylbenzidine; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; UHPLC-HRMS: ultra-high performance liquid chromatography-high resolution-mass spectrometry; VIP: variable of importance in projection; OPLS-DA: Orthogonal Partial Least Square Discriminant Analysis.

This trial was registered at [clinicaltrials.gov](http://clinicaltrials.gov) as NCT02295878.

**Names for PubMed indexing:** Baldrick, McFadden, Ibars, Sung, Moffatt, Megarry, Thomas, Mitchell, Wallace, Pourshahidi, Ternan, Corona, Spencer, Yaqoob, Hotchkiss, Campbell, Moreno-Rojas, Cuevas, Pereira-Caro, Rowland, Gill.

## ABSTRACT

**Background:** Epidemiological evidence suggests a diet rich in (poly)phenols has beneficial effects on many chronic diseases. Brown seaweed is a rich source of (poly)phenols

**Objective:** The aim of this study was to investigate the bioavailability and effect of a brown seaweed (*Ascophyllum nodosum*) (poly)phenol extract from on DNA damage, oxidative stress, and inflammation *in vivo*.

**Design:** A randomised double-blind placebo-controlled crossover trial was conducted in 80 participants aged 30-65 years with a BMI  $\geq 25\text{kg/m}^2$ . The participants consumed either a 400 mg capsule containing 100 mg of seaweed (poly)phenol and 300 mg maltodextrin or a 400 mg maltodextrin placebo control capsule daily for an 8-week period. Bioactivity was assessed with a panel of blood-based markers including lymphocyte DNA damage, plasma oxidant capacity, C-reactive protein and inflammatory cytokines. To explore the bioavailability of seaweed phenolics, an untargeted metabolomics analysis of urine and plasma samples following seaweed consumption was determined by UHPLC-HR-MS.

**Results:** Consumption of the seaweed (poly)phenols resulted in a modest decrease DNA damage but only in a subset of the total population who were obese. There were no significant changes in CRP, antioxidant status, inflammatory cytokines. We identified phlorotannin metabolites that are considered potential biomarkers of seaweed consumption including pyrogallol/phloroglucinol-sulfate, hydroxytrifuranol A-glucuronide, dioxinodehydroeckol-glucuronide, diphlorethol sulfates, C-O-C dimers of phloroglucinol sulfate, C-O-C dimers of phloroglucinol and diphlorethol sulfate.

**Conclusion:** To the best of our knowledge, this work represents the first comprehensive study investigating the bioactivity and bioavailability of seaweed (poly)phenolics in human participants. We identified several potential biomarkers of seaweed consumption. Intriguingly, the modest improvements in DNA damage were observed only in the obese subset of the total

74 population, the subgroup analysis should be considered exploratory as it was not preplanned;  
75 therefore, are not powered adequately. Elucidation of the biology underpinning this observation  
76 will require participant stratification according to weight in future studies.

77

78 *Key words:* Seaweed, Phenolic compounds, Inflammation, DNA damage, Oxidative stress,  
79 bioavailability

80

## INTRODUCTION

Diets rich in plant-derived foods may reduce risk of chronic degenerative diseases, including cardiovascular disease (CVD), with the beneficial effects being attributable in part to highly bioactive (poly)phenolic compounds contained therein (1-3). Fruits and vegetables are well-known sources of (poly)phenols, but a less well-known source is *Ascophyllum nodosum*, a brown algae common to the British Isles that is rich in (poly)phenolic compounds, including, uniquely, phlorotannin (4, 5). Phlorotannins are oligomers of phloroglucinol whose concentration in seaweed is affected by numerous factors including plant size and age, water salinity, nutrient and heavy metal content, in addition to light intensity changes (6-8). *Ascophyllum nodosum* is of interest as it is one of only a few commercially sustainable seaweed species.

Phlorotannins – and brown seaweed extracts in general – exhibit beneficial effects on a range of biological processes including modulation of inflammation, reduction of oxidative stress and improvements in cardiovascular function (9-11). However, the evidence base relies heavily on cell line and small animal models, with few studies to date involving humans (12-13). Phlorotannin-rich extracts from brown seaweeds have been shown to be effective in controlling inflammation *via* a number of pathways including inhibition of pro-inflammatory cytokines including tumour necrosis factor (TNF)- $\alpha$  and interleukins (IL)-1 $\beta$  and IL-6 *in vitro* (14). The efficacy of *Ascophyllum nodosum* and other Furoid species extracts in mitigating the effects of oxidative stress by inhibiting the generation of reactive oxygen species (ROS), preventing DNA damage and in stimulating the production of glutathione in affected cells has been demonstrated in our work and that of other researchers (15-20). Our initial *in vitro* (15) and acute *in vivo* (20) observations of the antioxidant and anti-inflammatory activities of *Ascophyllum nodosum* extract(s) gave rise to the hypothesis that longer term consumption of *Ascophyllum nodosum*-derived (poly)phenols would be of benefit *in vivo*.

A few investigations have evaluated – either *in vitro* or *in vivo* – the bioactivity of phlorotannin rich extracts from Furoid species. For example, safe consumption levels for a (poly)phenol-enriched extract of the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus* and its effects on glycaemic response have been determined in clinical trials (21). To the best of our knowledge, we now report the first clinical study aimed at specifically addressing the effects of a phlorotannin-rich extract from *Ascophyllum nodosum* on oxidative damage to DNA, plasma antioxidant capacity, inflammatory responses and chronic, low level inflammation *in vivo*.

## **PARTICIPANTS AND METHODS**

### **Seaweed material**

Fresh *Ascophyllum nodosum* was supplied by The Hebridean Seaweed Company, Isle of Lewis, Scotland, UK, in March 2011. The seaweed biomass was harvested by hand to ensure quality, cleaned of contaminating sand and fouling organisms and then shipped refrigerated to the processing facility (CEVA) in France where it was immediately chopped and frozen.

### **Preparation of food-grade seaweed extracts and capsule**

A (poly)phenol-rich seaweed extract from *Ascophyllum nodosum* was produced by CEVA (France) using a food-grade solvent (ethanol:water, 60:40 vol/vol) extraction system that was specifically developed for use with fresh or frozen *Ascophyllum nodosum*. Approximately half of the produced extract was then fractionated using tangential flow ultra-filtration to produce further extracts of varying molecular weight ranges and with varying (poly)phenol content. A standardised blended (poly)phenol-rich *Ascophyllum nodosum* extract was formulated by CEVA (**Table 1**) using 175 mg of extract and 50 mg of high molecular weight fraction (>10 kDa cut off) for use in the current study, in order to maximise the seaweed (poly)phenol content

(>100 mg per day) available from the extraction of fresh or frozen *Ascophyllum nodosum* against the need to minimise the level of iodine to within accepted regulatory guidelines (<500 µg per day) (**Table 1**), heavy metal contamination was also assessed. Maltodextrin (175 mg) was added to the capsule formulation as an excipient. Blending was carried out at the food-grade CEVA facilities in France. Samples of 400 mg of the *Ascophyllum* (poly)phenol-rich blend (SPE) or a placebo containing 400 mg maltodextrin only were packed into identical white, opaque, vegetarian capsules by Irish Seaweeds, Belfast, UK and identically sized and matched capsules were used for the clinical study. The food-grade seaweed capsule was characterized by NP-HPLC and LC-MS analysis and is reported elsewhere (20) (see **Supplemental figures 1 & 2**). Phlorotannins were quantified using the Folin-Ciocalteu Method (22) using phloroglucinol as the standard. In brief, 1 mL of suitability diluted sample was reacted with 1 mL of 40 % Folin Ciocalteu reagent for 5 min, and then made alkaline with the addition of 1 mL of 100 g/L Na<sub>2</sub>CO<sub>3</sub>. Absorbance was read at 730 nm after the solution had developed for 1 h at room temperature. Phloroglucinol dihydrate (range 0-30 mg/L) was used as a standard and was treated in the same way as samples.

## **Ethics and participants**

Ethical approval was received from the Ulster University Research Ethics Committee (UUREC). All participants gave written informed consent. Participants were recruited between May 2011 and August 2011 from Ulster University and the surrounding area. The intervention study ran between August 2011 and February 2012. The study was registered at [clinicaltrials.gov](http://clinicaltrials.gov) as NCT02295878.

The study was conducted in 80 participants (age range 30-65 years). All participants were apparently healthy, non-smoking, BMI $\geq$ 25 kg/m<sup>2</sup>, omnivores, who did not habitually use vitamin or mineral supplements, as determined using a pre-screening health and lifestyle



questionnaire. Pregnant and lactating women, vegetarians and vegans and lactose-intolerant individuals were excluded from the study, as were those with chronic medical complications such as diabetes, cardiovascular disease, autoimmune/inflammatory disorders, or who had chronic medication use including anti-inflammatory agents.

## **Study design**

The study was a 24-week randomised, double-blind, placebo-controlled crossover trial. After obtaining consent, participants were randomly assigned, in blocks of four using a random-number generator ([www.randomization.com](http://www.randomization.com)), to either the intervention or the control. In total, eighty participants were randomised to 2 groups of 40, each starting on either a 400 mg seaweed (poly)phenol extract (SPE) capsule containing 100mg of (poly)phenols or a 400 mg maltodextrin placebo control capsule (Avebe MD14P) daily for an 8-week period. The participants were supplied with all capsules in weekly labelled capsule boxes at the beginning of each phase, which was interspersed by an 8-week washout phase. During the washout phase, the participants were asked to maintain their habitual diet. Participants were asked to bring any unconsumed capsules to their study appointment at the end of each treatment phase and were also contacted weekly by the study researcher to encourage compliance and to discuss any difficulties they were experiencing.

## **Blood and urine sample collection**

Fasting blood samples were collected before and after each phase (week 0, week 8, week 16, and week 24) by venepuncture into EDTA, serum or sodium heparin-containing tubes, as required. All blood samples were processed on ice. Lymphocytes were isolated by using Histopaque-1077, according to the manufacturer's instructions (Sigma Diagnostics, St Louis, MO), and plasma samples were prepared by centrifugation at  $1000 \times g$  for 10 min at 4 °C.

Serum samples were allowed to clot for 30 min at room temperature and then were centrifuged at  $2000 \times g$  for 10 min at 4 °C. Whole blood from sodium-heparin treated tubes was prepared according to the manufacturer's instructions (BD Bioscience Fast Immune Cytokine System). Whole blood, plasma, serum and urine samples were immediately stored at -80 °C, whereas lymphocytes were stored frozen in liquid nitrogen. All biological measurements were carried out at the end of the intervention in batches containing equal numbers of active and control phase samples in each batch, and the researchers were blinded to these samples during analyses. A 24 h urine collection occurred at each time point; volume and pH were measured and the sample mixed following which  $2 \times 14\text{ml}$  aliquots were removed and centrifuged at  $1000 \times g$  for 10 min at 4 °C. Supernatants were stored at -80 °C until required.

## **Questionnaire assessments**

All participants completed a health and lifestyle questionnaire assessing their alcohol intake and physical activity levels, as well as a validated 4-day food diary at the mid-point during each treatment phase (active/placebo) of the study. Data on type of food and corresponding weight was entered into a food analysis database (WISP, Weighed Intake Software Program; Tinuviel Software, Warrington, U.K.) by two independent researchers and the dietary composition calculated.

## **DNA damage in peripheral blood mononuclear cells**

Peripheral blood lymphocytes, previously isolated and stored in liquid nitrogen, were thawed and screened for basal single strand breaks (SBs) in DNA using the single cell gel electrophoresis (Comet) assay (23) as adapted by Gill *et al.* (24). Spontaneous DNA SBs are associated with an altered cell function and are considered appropriate for the substantiation of health claims in the context of protection against generic DNA damage (25). In addition,

resistance to induced DNA damage (SB) was measured in lymphocytes subjected to increased oxidative insult *ex vivo* by pre-treating lymphocytes with 150  $\mu\text{mol H}_2\text{O}_2/\text{L}$  for 5 min at 4°C, before the measurement of SBs. The mean (percentage DNA in tail) was calculated from 50 cells per gel (each sample in triplicate) and the mean of each dataset was used in the statistical analysis.

### **Plasma total oxidative capacity**

Total oxidative capacity (TOC) measures total peroxide levels in plasma, by the reaction of endogenous peroxides with peroxidases, using tetramethylbenzidine (TMB) as the chromogenic substrate (26) with spectrophotometric measurement at 450 nm. To 10  $\mu\text{L}$  of standard (freshly-prepared hydrogen peroxide, 0.1 mmol/L) or samples in an uncoated microtiter plate was added 200  $\mu\text{L}$  of reaction mixture (0.05M phosphate-citrate buffer, pH 5.0; TMB solution (1 mg/mL), and peroxidase (>2500U/mL) in a proportion of 100:10:1. Plates were incubated at room temperature for 15 min, following which 50  $\mu\text{L}$  of stop solution (2M  $\text{H}_2\text{SO}_4$ ) was added to all wells and absorbance at 450 nm was measured using a microplate reader (GENIOS Tecan). Hydrogen peroxide standard solutions (range 0 - 1 mmol/L) were freshly prepared before use.

### **Lipid profile and serum C-reactive protein**

Plasma total cholesterol, HDL cholesterol and triglycerides were measured on an Instrument Laboratory (ILAB) 600 (Warrington, UK) autoanalyzer using commercial kits (Roche diagnostics, Lewis, UK) according to kit manufacturer's protocols. Plasma LDL cholesterol was calculated using the Friedewald formula (27).

C-reactive protein was determined on an ILAB 600 autoanalyser using a *Quantex* CRP Ultra-Sensitive commercial kit (0.4-18.3 µg/dL) in accordance with the manufacturer's instructions.

### **Measurement of inflammatory markers**

Intracellular cytokine levels in lymphocyte and monocyte populations and tissue factor (TF) expression were assessed using a whole blood labelling method that utilises flow-cytometry (Fast Immune Cytokine System, BD Biosciences) in accordance with manufacturer's instructions for all participants at all time-points. The method was used to measure intracellular IL-1 $\beta$ , IL-2, IL-6, IL-10, IL-12, interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$  expression in mononuclear cells. Briefly, whole blood was incubated with either lipopolysaccharide or phorbol 12-myristate 13-acetate to activate monocytes and lymphocytes, respectively. Cells were labelled with the appropriate cell surface antibody and cytokine-specific antibody and analysed on a Gallios flow cytometer (Beckman Coulter). The number and percentage of each cell type expressing the cytokine, as well as the mean channel fluorescence was recorded. The cytokine profiles were examined by ratio of TNF- $\alpha$  to IL-10, IL-1 $\beta$  to IL-10, IL-6 to IL-10 and CRP to IL-10, according to Laird *et al.* (29).

### **Extraction of (poly)phenols from urine and blood samples.**

Urine samples were defrosted, vortexed, centrifuged at 16,110  $\times$  g for 10 min at 5 °C, and passed through 0.45 µm filter discs prior to the analysis of 50 µL aliquots by UHPLC-HR-MS. The extraction of metabolites from the plasma samples was carried out as described previously (28). Briefly, plasma samples were defrosted, vortexed and 400 µL aliquots mixed with 10 µL of ascorbic acid (10%, v/v), and 980 µL of 1% formic acid in acetonitrile. One µg of rutin was added to the samples as internal standard for plasma extraction efficiency. The samples were

then vortexed for 1 min and ultrasonicated for 10 min. After centrifugation at 16,110 g for 15 min, supernatants were reduced to dryness in vacuo using a concentrator plus (Eppendorf, Hamburg, Germany) and resuspended in 150  $\mu$ L of distilled water containing 1% formic acid and 50  $\mu$ L of methanol, which was then centrifuged at 16,110 g for 10 min. Analysis of 10  $\mu$ L aliquots of the supernatant was by UHPLC-HR-MS. Recovery of the internal standard was  $\pm 16\%$  (n=78).

#### **Non-targeted analysis of urine and plasma by UHPLC-HR-MS.**

Plasma and urine samples were analysed using a Dionex Ultimate 3000 RP UHPLC system comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Thermo Scientific). The HPLC conditions were previously described by Corona *et al.*, (20) with some modifications. Briefly, reverse phase separations were carried out using a 100 x 2.1 mm i.d. 1.8  $\mu$ m Zorbax SB C18 (Agilent) maintained at 25 °C and eluted at a flow rate of 0.2 mL/min with a 50 min gradient of 3-70% of 0.1% acidic methanol in 0.1% aqueous formic acid. After passing through the flow cell of the PDA detector the column eluate was directed to an Exactive<sup>TM</sup> Orbitrap mass spectrometer fitted with a heated electrospray ionization probe (Thermo Scientific) operating in negative ionization mode. Analyses were based on scanning from 100 to 1000 m/z, with in-source collision-induced dissociation at 25.0 eV. The capillary temperature was 350 °C, the heater temperature was 150 °C, the sheath gas and the auxillary gas flow rate were both 25 and 5 units, respectively, and the sweep gas was 4 and the spray voltage was 3.00 kv. Data acquisition and processing were carried out using Xcalibur 3.0 software.

Untargeted analysis of the selected urine and plasma samples was performed using mass spectral data from the orbitrap analysis applied to the Compound Discoverer software (version 2, Thermo Fisher Scientific Inc.). The Compound Discover application processes the raw data

into workflows that can be defined on the basis of the nature of the experimental setup. In our case, the workflow selected was 'untargeted metabolomics workflow' that includes retention time alignment, component detection, grouping, elemental composition prediction, gap filling, hide chemical background (using blanks), ID using mzCloud and ChemSpider and differential analysis. The parameters were adjusted to our experimental conditions. Samples were grouped and labelled according to our experimental design, either before or after supplementation of seaweed capsule, the output, as peak areas for the detected peaks was used to develop a multivariate data analysis by Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA). Analysis of seaweed phenolics in blood and urine was also undertaken by HPLC-DAD analysis (**Supplemental Tables 1 & 2**) in a manner consistent with previous studies (15, 20).

## **Power calculations and statistical analyses**

Power calculations were performed for the primary endpoint of the change in DNA damage in peripheral blood mononuclear cells. Based on data from a previous study (24), 72.6 participants were needed to detect a 25% change in DNA damage in lymphocytes ( $\alpha$  0.05).

All values are expressed as mean  $\pm$  SD, unless otherwise specified. The mean values are reported for all participants (n=78) during both treatment phases (SPE, Maltodextrin). Significant associations between several outcomes and confounders including BMI and gender were identified at baseline using bivariate correlations or independent t-tests, as appropriate (TOC & Gender P=0.03, DNA damage & BMI P=0.048, DNA damage & Gender P=0.009). Consequently, data was also analysed by stratification of increasing risk, including overweight participants (n=42) and obese participants (n=36). The subgroup analysis was not preplanned; therefore, are not powered adequately and should be considered exploratory. All biochemical analysis was conducted in duplicates, unless otherwise stated, and the mean values taken as the final result. For all markers, the results are presented as treatment effects. This was undertaken

by calculating individual differences between pre- and post- values for both control and treatment phases for each subject. Paired T-tests were then carried out on the difference scores (post-treatment value *minus* pre-treatment values) for both treatment phases (SPE and Maltodextrin). Significance level was set at  $P < 0.05$ . All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows version 22.0 (SPSS Inc., Chicago, IL, USA).

### **Multivariate Data Analysis**

Data were obtained as peak areas from the Compound Discover automatic integration software and consisted of 2194 and 3289 potential metabolites (or features) in urine and plasma samples, respectively. Relative peak areas of the metabolites (normalized by the total urine excretion of each subject) obtained by UPLC-HRMS were imported into MATLAB R2015b (Mathworks, USA). PLS toolbox v.8.5 (Eigenvector, USA) and homemade scripts were used. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) are the most widely used tools to explore similarities and patterns among samples where data grouping are unclear. Moreover, the orthogonal partial least squares discriminate analysis (OPLS-DA) method was performed as a typical supervised multivariate methodology used in metabolomics studies (30, 31). Several data pre-processing transformations were performed and evaluated, such as Probabilistic Quota Normalization (PQN), Log transformation, mean centering, pareto scaling and auto-scaling. In our case, PQN and autoscaling were selected as pre-processing techniques to reach the lowest root mean square error (RMSE) in an iterative process. A re-sampling method, cross-validation k-fold cross validation ( $k=5$ ) was also used to evaluate the number of latent variables (according to the lowest RMSE) and the prediction ability of our models. To reduce the impact of the random split of CV-participants, the mean values of the estimated results were obtained after 20 random 5-fold CV. Urine models provided successful

classification results at the cross validation step and achieved good prediction parameters that can be explained by the area under the curve values (1.00 and 0.895). Based on the plasma data set and classes selected a discriminant model could not be developed. Moreover, the Variable Importance in Projection (VIP) is the widely known metric that is used to identify potential markers in metabolomics studies (32). VIP is a weighted sum of squares of the PLS weight which indicates the importance of the variable to the whole model. The cut off VIP value selected in our study was 2. The potential markers were extracted from the two models to compare the results obtained.

## RESULTS

### Baseline characteristics

Eighty participants (males n=39, females n=41) were enrolled on this 24-week randomised, double-blind, placebo-controlled crossover trial. The intervention was conducted as per the protocol and there were no adverse events associated with the intervention. The study population had a mean group age of  $42.7 \pm 7.1$  years and a mean BMI of  $30.2 \pm 3.9$  kg/m<sup>2</sup>. The study had an overall compliance of 97% with 78 participants completing the 24-week study; two participants withdrew from the study at the midpoint for personal reasons and compliance was not significantly different by treatment group or time period ( $P > 0.05$ ) (**Figure 1**). There were no significant differences between the participants in age and physical characteristics at the beginning of either treatment phase (**Table 2**).

### Habitual dietary intake

Dietary analysis of habitual intake (midpoint) during both treatment phases (SPE, Maltodextrin) of the crossover trial is described in **Table 3**. There were no significant differences between treatment phases for any of the macronutrients or micronutrients analysed



indicating that the seaweed phenolic extract did not affect the habitual food consumption patterns in the study population.

### **Effects of seaweed (poly)phenol extract on DNA damage**

Basal levels of DNA damage observed in the study were consistent with previous studies with a mean group average of  $6.72 \pm 2.48\%$  tail DNA (data not shown). In response to an oxidative challenge with  $150 \mu\text{M H}_2\text{O}_2$ , DNA damage was increased to an average of approximately  $34 \pm 7\%$  tail DNA in both the placebo and SPE phase in all participants (**Table 4**). The 8-week intervention with a seaweed phenolic extract resulted in a significant reduction in basal DNA damage, as measured by the Comet assay, in obese participants only ( $\text{BMI} > 30 \text{ kg/m}^2$ ), with a significant reduction ( $P=0.044$ ) in basal DNA damage observed (**Table 4**). A significant reduction was not observed in the total population ( $n=78$ ) nor in participants classified as overweight ( $n=42$ ), either in terms of a challenge response or in basal levels. In addition, we noted that consumption of seaweed phenolic extract also significantly reduced ( $P=0.009$ ) basal DNA damage in males only with a mean group change value of  $-0.8 \pm 2.5\%$  tail DNA (SPE) compared to  $0.9 \pm 2.8\%$  tail DNA in the control. No significant effects were observed for females.

### **Total oxidative capacity of seaweed phenolics**

Total oxidative capacity (peroxide levels) in plasma samples from all participants ( $n=78$ ), in overweight participants ( $n=42$ ) and in obese participants ( $n=36$ ) were measured after the placebo and seaweed capsule intervention (**Figure 2**). There were no significant changes from baseline after either treatment phase (SPE, Maltodextrin) in all participants ( $n=78$ ) nor in overweight ( $n=42$ ) or obese ( $n=36$ ) sub groups. However consumption of seaweed phenolic extract also significantly reduced ( $P=0.018$ ) TOC in females only with a mean group change

value of  $-7.44 \pm 29.37 \mu\text{M}$  peroxides (SPE) compared to  $4.33 \pm 22.36 \mu\text{M}$  peroxides in the control. No significant effects were observed for males.

### **Effects of seaweed (poly)phenol extract on blood lipids and CRP**

Analysis of Pre- and post- values, and the percentage change, for both the placebo phase and the SPE phase for each blood lipid biomarker and CRP indicated that the 8-week supplementation with seaweed (poly)phenol extract did not significantly affect any cardiovascular risk marker (**Table 4**).

### **Effects of seaweed (poly)phenol extract on inflammatory markers**

No significant differences were measured in any of the inflammatory cytokines in either treatment phase (SPE, Maltodextrin) for all study participants (n=78) (**Figure 4**) and in addition no significant effects on the cytokine profiles were observed.

### **Bioavailability of seaweed phenolics, untargeted analysis**

Initially an unsupervised PCA model was carried out (**Figure 4A**) on the urine data from all 78 participants. The first component of the PC1 vs PC2 scores plot obtained from the data set explained 23.14% of the total variance showing a clear trend among some of the participants compared to the rest. Moreover, a hierarchical cluster analysis was performed to verify this natural aggrupation pattern among the 78 participants suggesting, two distinct clusters among all the participants (**Figure 4B**), accounting for the inter-individual differences associated with variation in the urinary metabolite profiles. Recent studies suggest that individuals can be clustered into distinct groups based on their gut microbiome composition, functional metabolism (33) or individual responses to obtain markers of a specific treatment, minimizing the inter-individual data that are not the target of the study. In order to elucidate the observed

variability between these two groups, two supervised models were performed, one with the urinary profiles from all the participants before the seaweed consumption and another one with the urinary profiles from all the participants after seaweed consumption. Both supervised models verified differences between the two groups of individuals (Group 1 and Group 2) and provided successful classification results at the cross validation step and achieved in the pre-treatment and post-treatment samples 95% and 98% sensitivity (participants of the class of interest correctly assigned to their class) and 100% and 100% specificity (participants not belonging to the class of interest were correctly not assigned to that class), respectively (**Supplemental Figure 3**). It is noteworthy that even stratifying for BMI category, the multivariate analysis of the urine profiles did not show clear differentiation of the metabolome between obese or overweight individuals.

By using the preliminary information provided by PCA and HCA related to the stratification of the individuals into groups which share a common excretion profile before and after supplementation with seaweed capsules, two data sets were analysed. The individuals were stratified into two groups; Group 1 including 70 participants; and Group 2 including 8 participants (S58, S60, S61, S71, S76, S78, S79, S83, all being overweight individuals). Two supervised OPLS-DA models were built to discriminate according to the seaweed treatment in both models (**Figure 5**). In our study we used two latent variables (LV) and the sensitivity and specificity values were set to 100% at the calibration step recognition ability of the two models. These analyses showed that the human urine metabolome in both groups of participants was modified after the 8-week supplementation with seaweed (poly)phenol extract compared to baseline urine metabolic profiles. Using the loadings plot and the variable importance in projection values (VIP) we ascertained important contributors between the modelled classes and therefore identified the compounds responsible for the difference in the urine metabolic profile before and after 8-weeks seaweed (poly)phenol extract consumption in both groups.

VIP is the widely known metric that is used to identify potential markers (metabolites) in metabolomics studies. Further, the urine scores plot allowed the identification of those metabolites which appeared after the seaweed (poly)phenol extract intake (**Figure 5**) in both groups of participants.

The main contributors to metabolome differentiation in the urine before and after seaweed consumption in both groups of participants are described in **Supplemental Tables 3 & 4**. Positive loading values (x axis) and higher VIP values ( $VIP > 2.0$ ) were detected in urine after seaweed consumption and were the responsible of the observed differences between the participants classes (post ingestion vs non ingestion of seaweed (poly)phenol extract). Urine metabolites tentatively identified as pyrogallol/phloroglucinol sulfate, hydroxytrifurahol A-glucuronide and dioxinodehydroeckol glucuronide are considered clear biomarkers of seaweed consumption in Group 1 while C-O-C dimer of phloroglucinol-sulfate, C-O-C-dimer of phloroglucinol, fucophloroethol-glucuronide, diphlorethol sulfates and dioxinodehydroeckol glucuronide are those biomarkers of seaweed consumption in Group 2. All of them have shown high values in the variable importance in the projection (values  $> 2$ ) (**Table 5**).

Based upon the identified seaweed (poly)phenol metabolites, the total amount of seaweed metabolites excreted in urine varied noticeably between participant ranging from 0.001 to 4.140 m moles, with a group average of  $1.29 \pm 0.88$  m moles. Some 25% of the population (19/78) appeared to be low excretors, with urinary seaweed metabolites excretion less than 0.5 m moles, while 55% of the population (43/78) were medium excretors (0.5 and 2 m moles) and the remaining 20% of the population (16/78) were high excretors ( $> 2$  m moles) (**Supplemental Table 5**). Despite exploration using PCA or HCA, the plasma metabolome did not allow a clear differentiation between participants in either treatment phase (data not shown). As a result, it

was not possible to define exposure biomarkers in plasma samples across all the participants (n=78).

## DISCUSSION

The association of seaweed consumption with reduced CVD risk factor has been tested largely within *in vitro* or animal models, with only limited human data allowing substantiation of the proposed beneficial properties of seaweeds (12, 13, 34). The work reported in this paper represents the first comprehensive study of the *in vivo* bioactivity and bioavailability of seaweed (poly)phenolics on biomarkers of inflammation and oxidative stress in overweight and obese individuals. Participant retention rates were high (98%) and self-reported SPE capsule intake (97%) indicated that the intervention was implemented successfully in this group of participants. There was, however, no significant difference between the intervention (SPE capsule) and control (placebo capsule) phases for markers of oxidative stress, antioxidant status or inflammation in the population as a whole (n=78). Stratifying for BMI however revealed a significant albeit modest decrease in DNA damage in the obese population (n=36). This is consistent with Park *et al.* (35) who tested the natural microalgal carotenoid astaxanthin, in 14 healthy females (2 mg extract/d for 8 weeks) and showed a significant reduction in the oxidative damage plasma marker 8-hydroxy-2'-deoxyguanosine. Obese individuals are associated with increased basal DNA damage, higher oxidant status and increased oxidative damage to macromolecules as a group and are thus at higher risk of chronic disease (36-39). In the current work, an overweight/obese population (age 30-65 years, BMI >25 kg/m<sup>2</sup>) supplementation with seaweed (poly)phenolics significantly reduced basal levels of lymphocyte DNA damage (by 23%) but only in the obese sub-population (n=36). These two seaweed-extract interventions are consistent with data from *in vitro* studies which demonstrated anti-genotoxic activity for seaweed extracts on a range of cell lines (40,41), including the SPE

extract used in our study (15). The biological mechanism underpinning the observed reduction in DNA damage in the obese group could not be determined from our data, however the activation of the cytoprotective Nrf2/ARE pathway may be involved, as seaweed (poly)phenols such as eckol are anti-genotoxic (42) and can activate Nrf2-mediated HO-1 induction (43,44) consistent with effects observed for (poly)phenols from terrestrial sources (45,46). We observed a 28% decrease in CRP levels, as a marker of inflammation, in response to seaweed extract consumption however this change was not significant, in contrast to the observations of Park *et al.* (35). However, a study investigating the effect of consuming *Palmaria palmata* (5 g/day) incorporated into bread found that it significantly increased CRP by 16%, suggesting that *P. palmata* stimulates inflammation rather than reducing it (47). Park *et al.* (35) reported that there was no difference in TNF and IL-2 concentrations, but plasma IFN- $\gamma$  and IL-6 increased on week 8 in participants given 8mg astaxanthin. Within our study, we similarly observed no significant changes in IL-2 or TNF, nor did we see an alteration in IFN- $\gamma$  or IL-6. The seaweed extract tested did not affect immune function (cell mediated and humoral immune responses as tested by the cytokine markers) following supplementation rather than reducing it (47). Park *et al.* (35) reported that there was no difference in TNF and IL-2 concentrations, but plasma IFN- $\gamma$  and IL-6 increased on week 8 in participants given 8mg astaxanthin. Within this study, we similarly observed no significant changes in IL-2 or TNF, nor did we see an alteration in IFN- $\gamma$  or IL-6. The seaweed extract tested did not affect immune function (cell mediated and humoral immune responses as tested by the cytokine markers) following supplementation.

A comparison of metabolic profiles between urine and plasma samples of 78 individuals who consumed seaweed capsules has allowed evaluation of seaweed molecules potentially responsible for the modest beneficial effects observed *in vivo* as well as identifying biological markers linked to seaweed consumption. We determined differences in the urine metabolite

profiles between participants, and thus were able to stratify the individuals into groups that shared common excretion metabolite profiles both pre- and post- seaweed supplementation. Despite substantial inter-individual variation in the concentration of seaweed metabolites excreted in urine (0.001 to 4.140 m moles, the urinary profiles of these two groups were statistically different and we have been able to select the metabolites responsible for this discrimination as potential biomarkers of seaweed consumption (**Table 5**). Our comprehensive multivariate analysis of the metabolite profiles showed the expected person-to-person variation in the 0-24h urinary excretion of seaweed (poly)phenols, which could be attributed to differences in gut microbiota or living conditions amongst study participants. Nonetheless, we could group the 78 participants by ability to metabolize seaweed (poly)phenols. Group 1 individuals were characterized by greater excretion of seaweed-derived metabolites such as pyrogallol/phloroglucinol sulfate, hydroxytrifurahol A-sulfate and dioxinodehydroeckol, while Group 2 individuals were characterized by excretion of C-O-C dimer of phloroglucinol-sulfate, C-O-C dimer of phloroglucinol, fucophloroethol-glucuronide, diplorethol sulfates and dioxinodehydroeckol glucuronide. This inter-individual variation in absorption of seaweed polyphenols is consistent with profiles previously observed in the bioavailability of dietary (poly)phenols such as orange, cranberry and pomegranate juices (48-50).

The seaweed phenolic metabolites and can be divided into (a) phase II sulphated and glucuronidated metabolites related to the targeted components described earlier by Corona *et al.* (20) (**Supplemental Tables 1 & 2**); arguably formed in the liver, and (b) an extended list of unknown compounds which could be potential breakdown products or metabolites of the original seaweed (poly)phenols catabolised by colonic bacteria. However, further investigation would be required to confirm the identity and origin of these unknown compounds. We previously reported the poor absorption of the high molecular weight phlorotannins in the upper

gastrointestinal tract (20) likely results in them reaching the colon and becoming subject to microbial fermentation to lower molecular weight derivatives, as we have recently shown using an in vitro gut microbiota model (15). The urinary metabolite profiles of seaweed phenolics from the low and high excretors clearly indicated a high inter-individual variation in metabolism. It is possible that inter-individual variation in gut microbiota underlie these metabolic changes and were responsible for the observed differences (51-53). The study of seaweed polyphenol(s) bioavailability remains challenging due to the high range of molecular weight compounds present, and their characterisation is complicated further by the lack of commercially available standards. Other limitations of the present study include a relatively short-term intervention, and thus results cannot be extrapolated to long-term chronic consumption. In addition, study participants were mostly recruited from the University of Ulster staff and local residents and thus may not be representative of the general overweight population of Northern Ireland. While lymphocyte DNA damage (spontaneous DNA SBs) is considered appropriate for the substantiation of EFSA health claims in the context of protection against generic DNA damage, a lesion specific enzyme such as (ENDO III) would have been required for a claim related to oxidative DNA damage (54). With respect to the TOC assay it is based on the reaction of endogenous peroxides with hydrogen peroxide, using TMB as the chromogenic substrate, and provides a measure of total peroxide levels in plasma. While this assay lacks specificity in comparison to other measures of oxidative stress, the direct correlation between oxygen radicals and peroxides allows measurement and characterization of the oxidative status/oxidative stress in biological fluids. Moreover the test is characterized by linearity, good precision, and endpoint determination (55). Finally, the study population number may have been too small to yield significant results in intercellular cytokines. These factors should be considered for the design of future studies in this area.



In conclusion, this work represents the first comprehensive human study investigating bioavailability and metabolism of seaweed (poly)phenolics. Consumption of SPE decreased DNA damage—albeit to a modest extent—in obese individuals only, with no clear effects on clinical markers of inflammation. Untargeted analysis identified novel urinary biomarkers of seaweed consumption and highlighted a high degree of inter-person variation in the metabolism of seaweed phenolics. Future studies that address the ingestion of seaweed phenolics will need to consider and adjust for these parameters, and this work has highlighted the importance of establishing an individual’s capacity for metabolising (poly)phenols.

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Table 1. Key components of phlorotannin rich basic seaweed extract, High Molecular Weight (HMW) seaweed extract fraction, and blend used for intervention seaweed polyphenolic extract capsules (SPE).

<b>Extract component</b>	<b>Basic seaweed extract</b> mg/175mg of extract	<b>HMW seaweed extract fraction</b> mg/50mg of extract	<b>Blend (SPE capsule)</b> mg/400mg capsule
Phlorotannin	61.25	46.05	107.3
Iodine	0.48	0.02	0.5
Maltodextrin <sup>1</sup>	0	0	175
Minerals	39.38	13	52.38
Fucoxanthin	0	36.5	36.5
Laminarin as glucose	10.68	1.75	12.43
Fucoidan as fucose	0	<0.001	<0.001
Mannitol	29.23	5.9	35.13
Inorganic arsenic	<0.001	<0.001	<0.001
Cadmium (LD 0.15mg/kg)	<LD	<LD	<LD
Mercury (LD 0.016mg/kg)	<LD	<LD	<LD
Lead (LD 1.1mg/kg)	<LD	<LD	<0.001
Tin (LD 1.7mg/kg)	<LD	<LD	<LD

<sup>1</sup>maltodextrin was added to the capsule formulation as an excipient.

Table 2. Baseline characteristics of the study population (n=80).

Variable	Placebo	SPE
	treatment phase	treatment phase
	(n=80)	(n=80)
Age (y)	42.8 ± 7.2	42.9 ± 7.1
Gender (M/W)	20/20	19/21
Height (m)	1.71 ± 0.08	1.72 ± 0.10
Weight (kg)	88.9 ± 14.1	89.1 ± 17.3
BMI (kg/m <sup>2</sup> )	30.3 ± 3.5	30.0 ± 4.4

No significant differences  $P > 0.05$ , Paired T Test.

Table 3. Habitual nutrient intake of subjects during intervention study

Variable intake	Placebo	SPE	% Change <sup>2</sup>	P value <sup>3</sup>
	treatment	treatment		
	phase	phase		
	(n=77) <sup>1</sup>	(n=77)		
Energy (kcal/d)	1949 ± 590 <sup>4</sup>	2057 ± 684	5	0.110
Protein (g/d)	79.4 ± 28.6	79.9 ± 25.3	0.5	0.782
Carbohydrate (g/d)	220.6 ± 72.1	231.3 ± 84.2	5	0.278
Total fat (g/d)	78.6 ± 26.5	85.2 ± 34.8	8	0.058
Saturated fat (g/d)	29.0 ± 10.2	30.7 ± 15.3	6	0.417
Monounsaturated fat (g/d)	24.4 ± 9.3	26.8 ± 12.4	9	0.112
Polyunsaturated fat (g/d)	12.1 ± 5.4	13.5 ± 6.4	10	0.059
Fibre (g/d) <sup>5</sup>	12.9 ± 6.2	13.3 ± 4.8	3	0.259
Vitamin C (mg/d)	71.8 ± 50.5	69.4 ± 47.1	-3	0.909
Vitamin E (mg/d)	7.17 ± 3.7	7.67 ± 4.0	7	0.279
Folate (µg/d)	223.9 ± 98.3	228.2 ± 85.5	2	0.370
Carotene (µg/d)	2821 ± 1900	2553 ± 1706	-11	0.185

<sup>1</sup>S66 did not complete a food diary in either phase. <sup>2</sup> Calculated from SPE phase – Placebo

phase. <sup>3</sup> Mean treatment group values were not significantly different between phases, P<0.05

(Wilcoxin signed rank test). <sup>4</sup> Mean ± SD. <sup>5</sup>Calculated using the Englyst method.

Table 4. Effects of seaweed polyphenol extract on lymphocyte DNA damage, CRP and blood lipids.

Blood marker	Total (n=78)				Overweight (n=42)				Obese (n=36)			
	Average baseline value	Placebo treatment effect	SPE treatment effect	P value	Average baseline value	Placebo treatment effect	SPE treatment effect	P value	Average baseline value	Placebo treatment effect	SPE treatment effect	P value
DNA damage – basal (% Tail)	6.72 ± 2.48	0.74 ± 2.86	-0.41 ± 3.13	0.350	6.59 ± 2.80	0.32 ± 2.14	0.57 ± 3.24	0.129	6.91 ± 2.00	1.81 ± 4.50	0.15 ± 2.93	<b>0.044</b>
DNA damage - H <sub>2</sub> O <sub>2</sub> (%Tail)	34.2 ± 7.00	-1.56 ± 6.60	-2.03 ± 6.40	0.390	35.2 ± 7.11	0.13 ± 2.19	0.76 ± 1.66	0.062	32.8 ± 6.69	1.26 ± 2.09	0.54 ± 1.54	0.111
CRP (mg/ml)	2.67 ± 3.9	0.01 ± 3.3	-0.83 ± 4.9	0.429	2.59 ± 4.69	0.00 ± 3.40	-1.32 ± 6.22	0.348	2.80 ± 2.56	-1.81 ± 11.82	0.72 ± 5.15	0.258
Cholesterol (mmol/l)	5.20 ± 0.77	-0.06 ± 0.57	-0.10 ± 0.57	0.256	5.18 ± 0.82	0.01 ± 0.55	-0.53 ± 0.54	0.201	5.24 ± 0.73	-0.13 ± 0.58	-0.15 ± 0.60	0.419
Triglycerides (mmol/l)	1.51 ± 0.94	0.01 ± 0.82	0.04 ± 0.96	0.385	1.34 ± 0.72	0.07 ± 0.79	-0.04 ± 0.48	0.278	1.75 ± 1.15	-0.06 ± 0.85	-0.02 ± 1.34	0.497
HDL (mmol/l)	1.37 ± 0.32	-0.01 ± 0.15	0.03 ± 0.15	0.187	1.46 ± 0.31	0.01 ± 0.16	-0.03 ± 0.15	0.150	1.25 ± 0.32	-0.04 ± 0.11	-0.04 ± 0.14	0.446
LDL (mmol/l)	3.16 ± 0.1	-0.08 ± 0.5	-0.06 ± 0.50	0.478	3.13 ± 0.69	-0.07 ± 0.57	0.01 ± 0.45	0.383	3.20 ± 0.67	-0.09 ± 0.56	-0.13 ± 0.56	0.412

Data is presented as treatment affects, calculated based on individual differences between pre- and post- values for both control and treatment phases for each subject. Paired T-tests were then carried out on the difference scores (post-treatment value *minus* pre-treatment values) between treatment (Seaweed phenolic extract capsule) and placebo control phase (maltodextrin). Significance level was set at P<0.05 (one-tailed T test).

**Table 5.** Phlorotannins metabolites tentatively identified in human urine samples from Group 1 (70 subjects) and Group 2 (8 subjects) after seaweed capsule consumption.

<b>ID</b>	<b>VIP value</b>	<b>Rt (min)</b>	<b>Experimental Molecular Weight</b>	<b>Predicted Formula</b>	<b>Metabolite putative identification</b>	<b>Ratio<sup>1</sup></b>
<b>Group 1 (72 subjects)</b>						
652	2.06	25.6	205.9881	C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> S	Pyrogallol/phloroglucinol sulfate	1.5
800	2.28	25.8	205.9881	C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> S	Pyrogallol/phloroglucinol sulfate	1.2
1472	3.86	24.9	205.9881	C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> S	Pyrogallol/phloroglucinol sulfate	1.1
1352	4.26	25.6	486.1727	C <sub>22</sub> H <sub>30</sub> O <sub>12</sub>	Hydroxytrifuhaol A-glucuronide	2.0
1453	3.83	25.4	486.1727	C <sub>22</sub> H <sub>30</sub> O <sub>12</sub>	Hydroxytrifuhaol A-glucuronide	1.9
1458	4.23	25.3	486.1727	C <sub>22</sub> H <sub>30</sub> O <sub>12</sub>	Hydroxytrifuhaol A-glucuronide	2.2
1483	4.39	25.3	486.1727	C <sub>22</sub> H <sub>30</sub> O <sub>12</sub>	Hydroxytrifuhaol A-glucuronide	2.2
1917	2.11	31.4	544.2881	C <sub>27</sub> H <sub>44</sub> O <sub>11</sub>	Dioxinodehydroeckol glucuronide	1.1
<b>Group 2 (8 subjects)</b>						
293	2.08	9.5	327.0951	C <sub>12</sub> H <sub>8</sub> O <sub>9</sub> S	C-O-C dimer of phloroglucinol-sulfate	1.2
702	2.20	24.1	248.0315	C <sub>12</sub> H <sub>8</sub> O <sub>6</sub>	C-O-C dimer of phloroglucinol	1.2
853	2.08	27.8	797.3186	C <sub>45</sub> H <sub>49</sub> O <sub>13</sub>	Fucophloroethol glucuronide	1.1
1293	2.28	34.2	330.1675	C <sub>12</sub> H <sub>10</sub> O <sub>9</sub> S	Diphlorethol sulfate	1.9
1356	2.31	34.1	330.1675	C <sub>12</sub> H <sub>10</sub> O <sub>9</sub> S	Diphlorethol sulfate	1.7
1633	2.07	35.1	544.2152	C <sub>27</sub> H <sub>44</sub> O <sub>11</sub>	Dioxinodehydroeckol glucuronide	1.2

ID, identification number. VIP (variable influence in projection) is a variable that summarizes the importance of X variables to the OPLS-DA model. Variables with values > 2 were the most influential in the model. All predicted formula derived with < 5 ppm mass accuracy data.

<sup>1</sup>Ratio: seaweed capsule consumption/placebo consumption.

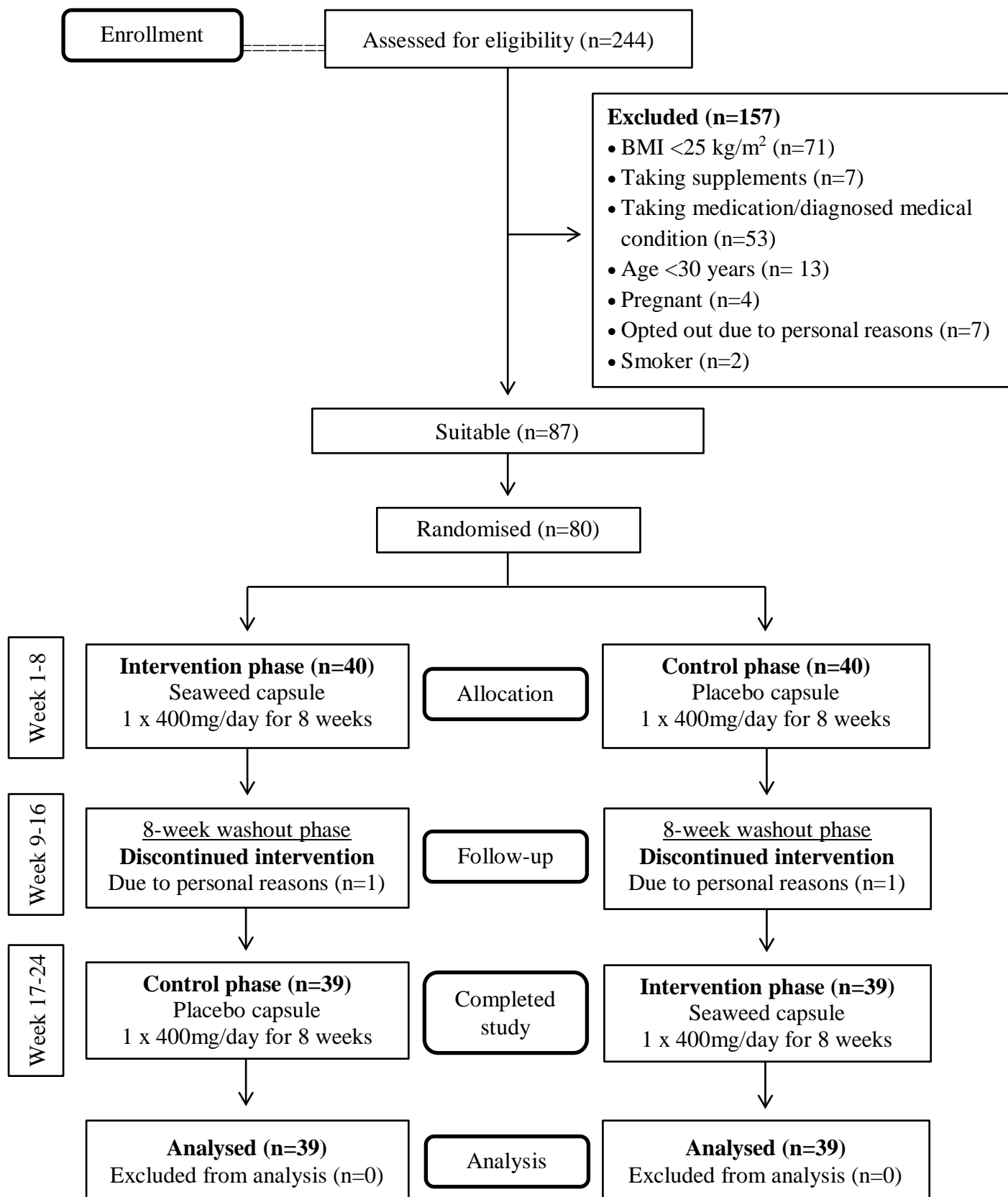
**FIGURE 1. CONSORT DIAGRAM.** Progress of participants through the intervention study.

**FIGURE 2.** Comparison of the effects of SPE supplementation phase (□) with that of a placebo phase (•) on total oxidative capacity (TOC) in the total study population (n=78), in overweight (n=42), and in obese (n=36). All values were measured in duplicate expressed as mean  $\pm$  SD. TOC is represented as the mean of the individual difference values (after – before supplementation) in the SPE and placebo treatment phases.

**FIGURE 3.** Comparison of the effects of SPE supplementation phase (■) with that of a placebo phase (□) on cytokine levels in the total study population (n=78), in overweight (n=42), and in obese (n=36). All values were measured in duplicate expressed as mean  $\pm$  SD. Cytokines were measured as the mean of the individual difference value (after – before supplementation) in the SPE and placebo treatment phases. Change in mean treatment group values were not significantly different between treatment phases,  $P > 0.05$  (Paired T Test; one-tailed test). M.F.I.; Mean Fluorescence Intensity.

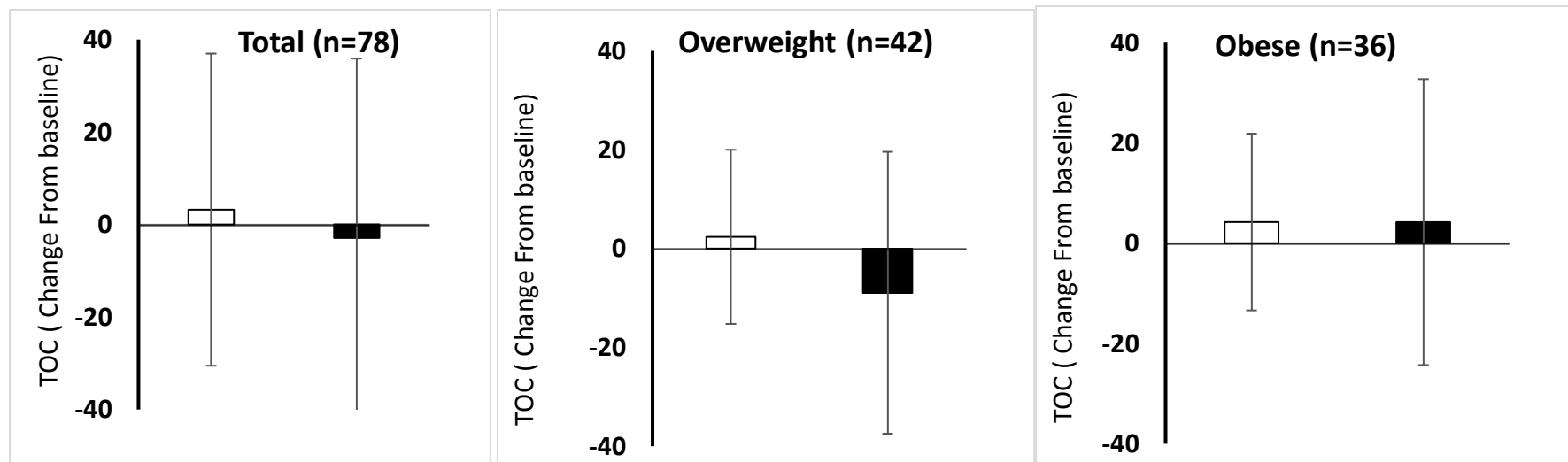
**FIGURE 4.** Principal Component Analysis (PCA) (A) and Hierarchical Cluster Analysis Cluster (HCA) (B) of urinary profiles before (♦) and after (■) seaweed consumption by 80 participants. The HCA was calculated based on Euclidean distances and the Ward hierarchical agglomerative method. The PC explained 23.14% of the total variance (PC-1 14.8% and PC-2 8.34 %).

**FIGURE 5** A) OPLS-DA scores and B) loadings of the urine samples belong to group of participants 1 (70 participants). C) OPLS-DA scores and D) loading of urine samples belong to group of participants 2 (8 participants) before (♦) and after (■) seaweed ingestion. (Circles shown in the graph represent a confidence of 95%). LV1: latent variable 1; LV 2: latent variable 2. The cut off VIP value selected to be 2. For VIP scores identification see **Table 5** and Supplemental **Table 1**.

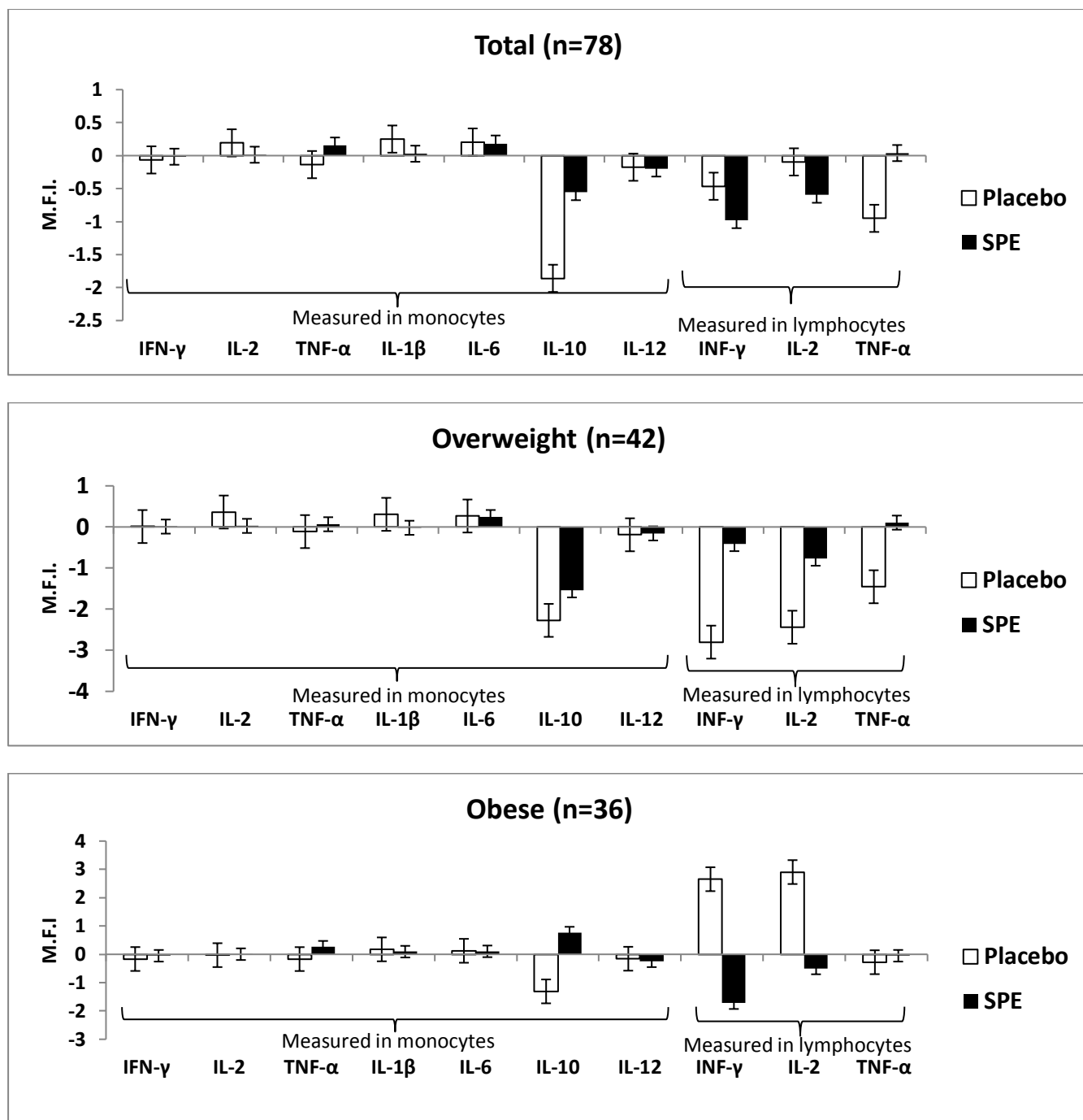


**FIGURE 1.**

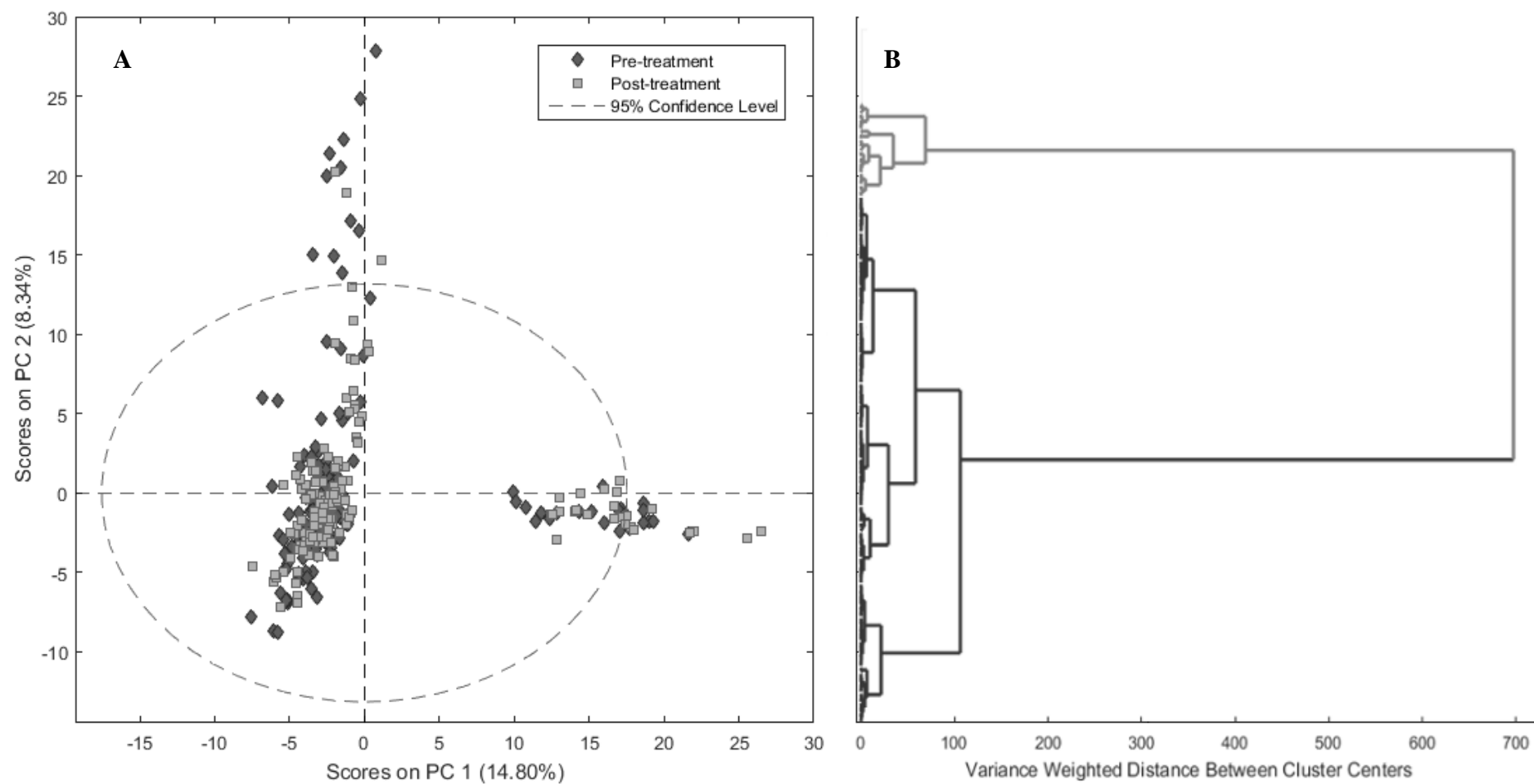




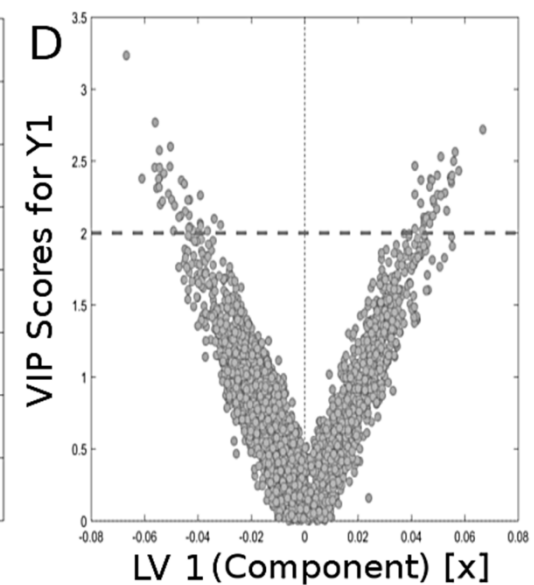
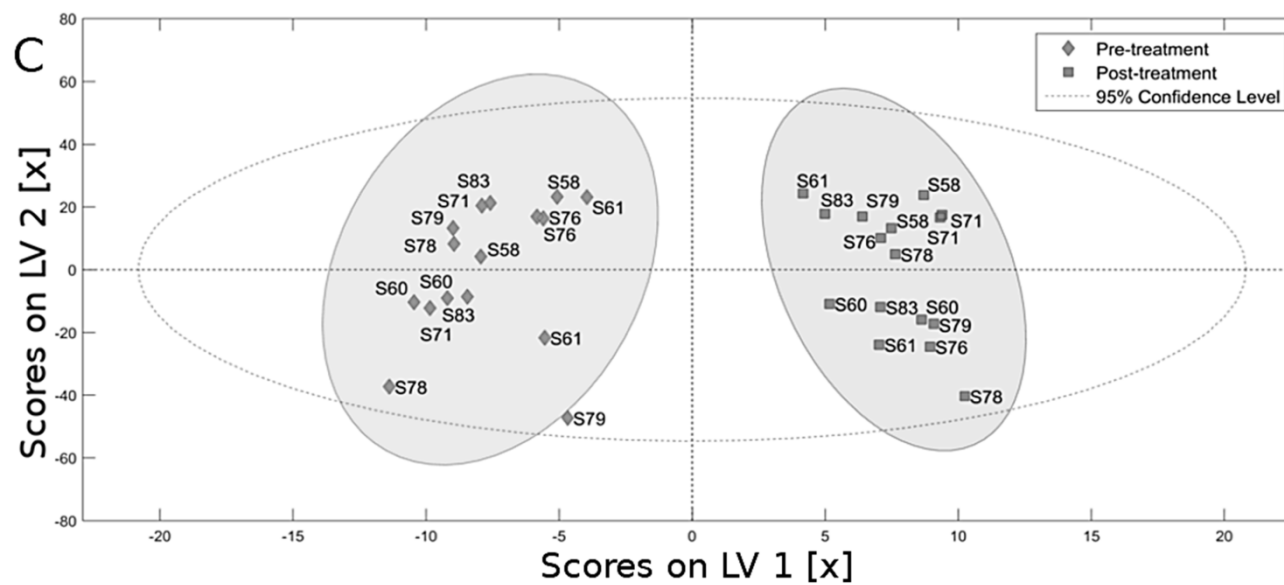
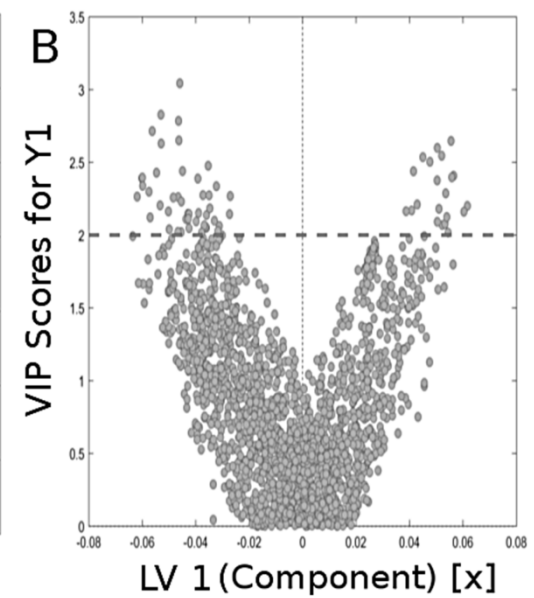
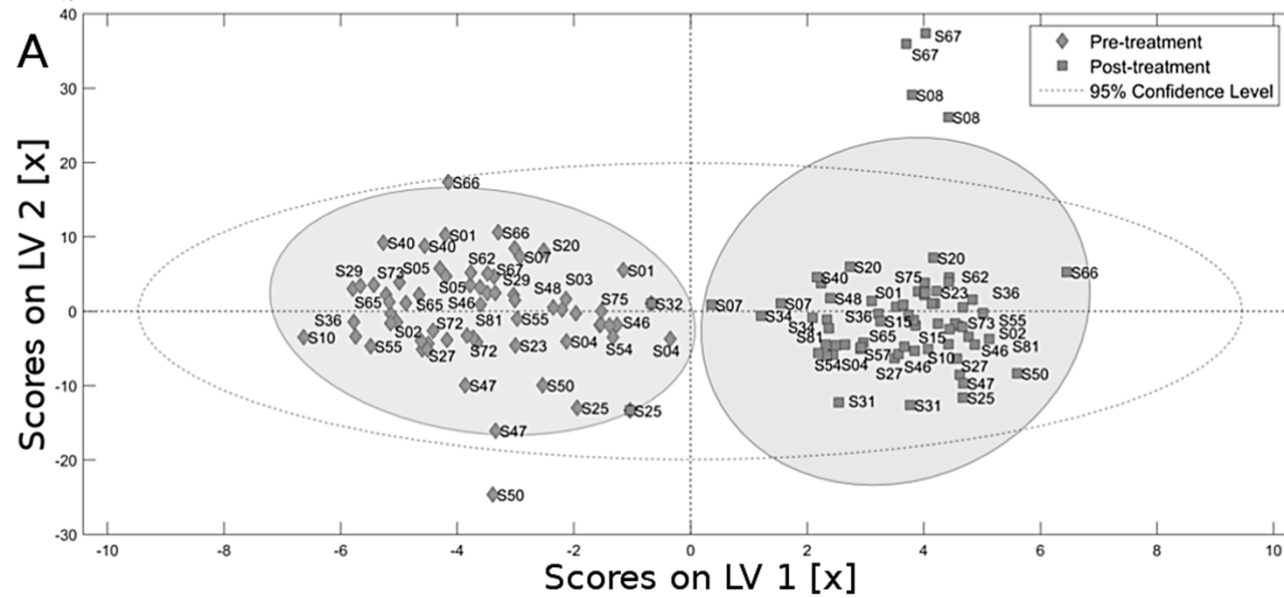
**FIGURE 2.**



**FIGURE 3.**



**FIGURE 4.**



## Supplemental Information

### **Impact of a polyphenol-rich extract from *Ascophyllum nodosum* on DNA damage and antioxidant activity in an overweight/obese population**

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<sup>f</sup>*Department of Food Science and Health, IFAPA-Alameda del Obispo, Córdoba, Spain*

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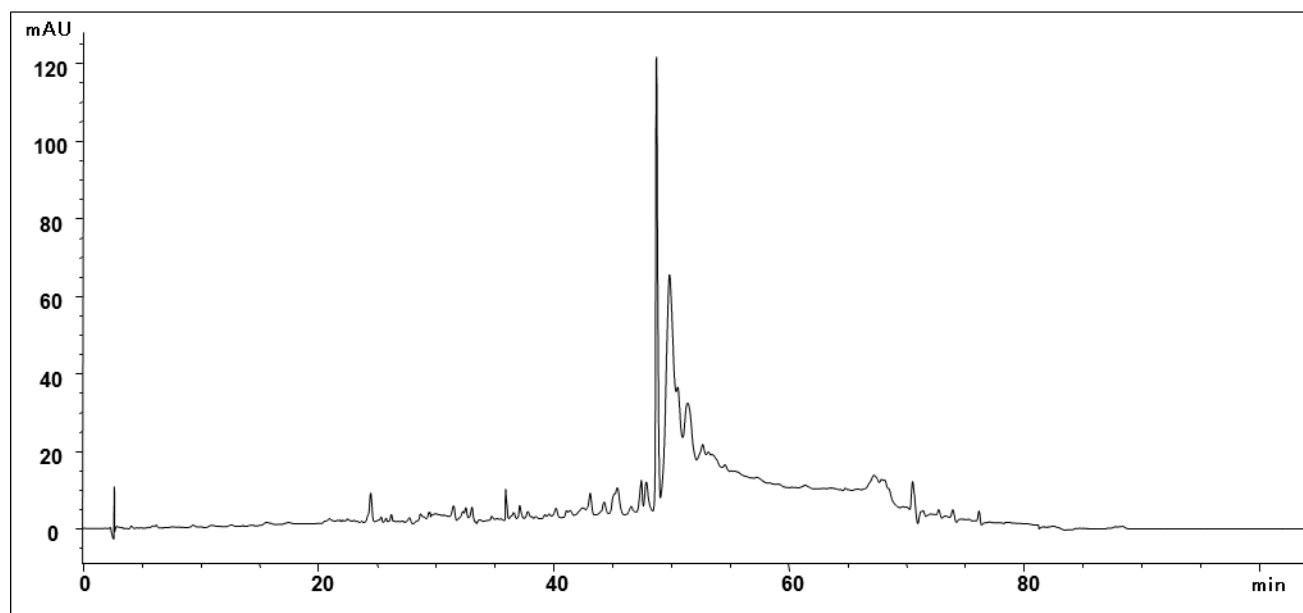
E-mail address: [c.gill@ulster.ac.uk](mailto:c.gill@ulster.ac.uk) (C. Gill)

**Analysis of the SPE capsule by NP-HPLC and LC-MS analysis** ( as reported in Corona et al Br J Nutr. 2016 Apr 14;115(7):1240-53.)

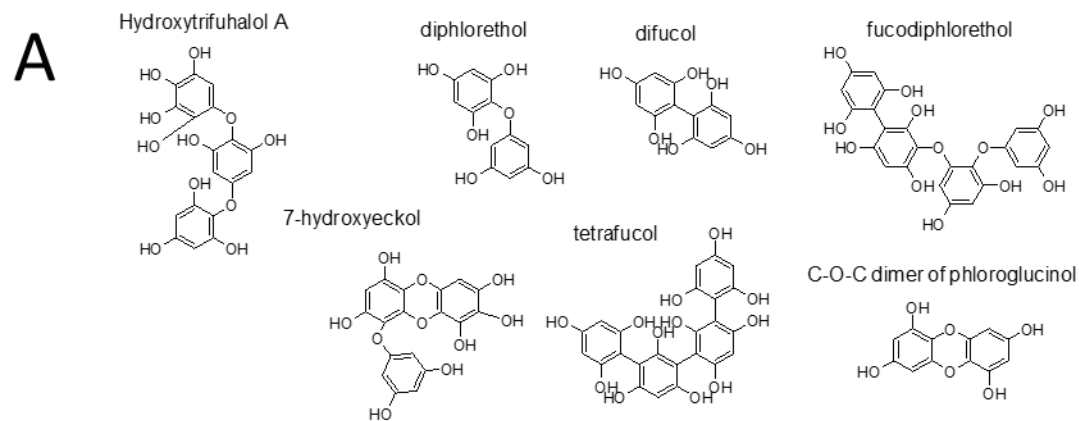
**NP-HPLC analysis:** The phlorotannins in the food-grade SPE used to produce the capsule were analyzed by normal phase HPLC analysis as previously described (Corona G et al, BJN, 2016) using an HPLC 1100 series equipped with LiChrospher Si60-5 column (250 mm × 4.0 mm ID, 5 µm particle size from HICHROM (LISP60-5-250AF), fitted with a guard column LiChrospher Si60-5 from HICHROM (LISP60-5-10C5). The mobile phase contained A: 82 % dichloromethane + 2 % methanol + 2 % acetic acid in water and B: 96 % methanol + 2 % acetic acid in water and was pumped through the column at 1 ml/min. 10 µl of samples were injected and analyzed by the gradient program which were (min/%B): 0/0, 30/17.6, 45/30.7, 50/87.8, 60/87.8, 80/0, 105/0 for detection of all compounds. The compounds were detected at a wavelength of 268 nm. All data were analyzed by ChemStation software. The phloroglucinol standard was injected at 0.1-100 µg/ml and phlorotannins in the capsules were analysed as phloroglucinol equivalents.

**LC-MS analysis:** LC-MS analysis was conducted to analyse the food grade seaweed capsule as previously described (Corona G et al, BJN, 2016), and was carried out in the negative ion mode using LC-MS/MS utilizing electrospray ionisation (ESI). Characterization was achieved using LC-MS/MS utilizing electrospray ionisation (ESI). This consisted of an Agilent 1200 HPLC system equipped with a binary pump, degasser, auto-sampler, thermostat, column heater, photodiode array detector and an Agilent 1100 Series LC/MSD Mass Trap Spectrometer. Separation of samples was achieved using a Zorbax SB C18 column (2.1 x 100 mm; 1.8 µm) (Agilent) and HPLC conditions were as follows: injection volume: 1 µL; column temperature: 25 °C; binary mobile system: (A) 0.1 % aqueous formic acid and (B) 0.1 % of formic acid in acetonitrile; flow rate: 0.2 mL/min. A series of linear gradients were used for separation (min/%B): 0/10, 3/10, 15/40, 40/70, 50/70, 65/10. Mass spectrometry was performed in the negative ion mode (scan range, m/z 100-1000 Da; source temperature, 350 °C). All solvents used were LC-MS grade.

The SPE (supplemental figure 1) comprised a wide range of molecular weights (20-70 min), with abundance of very high molecular weight phlorotannins eluting at later retention time (50-70min) in our Normal-Phase method. Using a RP-HPLC separation method coupled to ESI-MS analysis in negative ion mode (supplemental figure 3) we were able to identify some phlorotannin oligomers such as hydroxytrifuhalol A, tetrafucol, fucodiphlorethol, C-O-C dimer of phloroglucinol, 7-hydroxyeckol, diphlorethol and difucol.



**Supplemental Figure 1** . Chromatographic separation of phlorotannins contained in the seaweed extract by Normal-Phase HPLC with diode array detection (268nm).

**B****LC-MS analysis in negative ion mode of the seaweed extract phlorotannins**

Peak N	RT (min)	[M-H] m/z	MS <sup>2</sup> m/z		tentative identification
1	2.7	405	387	191	hydroxytrifuhalol A
2	3.3	497	479	353	tetrafucol, fucodiphlorethol
3	5.6	247	203		
4	10.6	387	369	230	C-O-C dimer of phloroglucinol
5	43.7	249	181	113	7-hydroxyeckol
6	46.2	249	181	113	diphlorethol / difucol

**Supplemental figure 2.** Characterisation of phlorotannins in the seaweed extract. A: Structures of phlorotannins identified in the seaweed extract: B: Phlorotannins in the seaweed extracts identified by LC-MS analysis in negative ion mode



### **Extraction /Analysis of Plasma and Urine samples by HPLC DAD.**

The HPLC-PDA described below has been removed from the original data due to the limitation of the HPLC DAD approach applied and replaced by analysis as described in paper. The data is now only available in supplemental information for context with existing papers on this topic using the same seaweed extract (SPE) .

- 1) Corona G, Ji Y, Anegboonlap P, Hotchkiss S, Gill C, Yaqoob P, Spencer JP, Rowland I. Gastrointestinal modifications and bioavailability of brown seaweed phlorotannins and effects on inflammatory markers. *Br J Nutr.* 2016 Apr 14;115(7):1240-53
- 2). Corona G, Coman MM, Guo Y, Hotchkiss S, Gill C, Yaqoob P, Spencer JP, Rowland I. Effect of simulated gastrointestinal digestion and fermentation on polyphenolic content and bioactivity of brown seaweed phlorotannin-rich extracts. *Mol Nutr Food Res.* 2017 Nov;61(11). doi: 10.1002/mnfr.201700223. Epub 2017 Aug 29

### **Extraction of (poly)phenols from urine and blood samples for HPLC DAD**

Urine samples were prepared as follows: 10 µl of internal standard solution (resorcinol 200 µg/ml) were added to 250 µl urine. Samples were analysed with and without enzymatic treatment (37°C, 40 min), in the presence of 1500 IU of β-glucuronidase and 50 IU of sulfatases from *Helix pomatia* (Type H-1). 1 ml of methanol acidified with 0.5% acetic acid was added, samples were mixed and centrifuged for 15 min at 16,100 x g at 4°C, supernatants were transferred

to a new tube and dried on a speedvac. Dried samples were resuspended in 125 µl of mobile phase, completely dissolved, centrifuged and transferred to vials for HPLC-DAD.

Plasma samples were prepared as follows: 10 µl of internal standard solution (resorcinol 200 µg/ml) were added to 450 µl of plasma, then 50 µl of 1.2 M acetic acid were added and samples were mixed. Samples were analysed with and without enzymatic treatment (37°C, 40 min) in the presence of 1500 IU of β-glucuronidase and 50 IU of sulfatases from *H. pomatia* (Type H-1). 1 ml of 100% methanol acidified with 0.5% acetic acid was added and samples were centrifuged for 15 min at 16,100 x g at 4°C and supernatants were collected. This step was repeated 3 times, the last occasion with 50% methanol acidified with 0.5% acetic acid, and the supernatants were dried using a speedvac. The pellets were dissolved with 125 µl of mobile phase and transferred to vials for HPLC analysis.

### **Analysis of seaweed phenolics in blood and urine by HPLC-DAD analysis**

Sample analysis was carried out with a Hewlett-Packard 1100 series liquid chromatography system (Hewlett-Packard, Palo Alto, CA) equipped with a diode array detector (HP Chem Station Software system). Samples were analysed by reverse-phase HPLC using a Nova-Pak C18 column (4.6 x 250 mm) with 4µm particle size. The temperature of the column was maintained at 30°C. The mobile phases consisted of a mixture of aqueous methanol 5% in 0.1% hydrochloric acid 5 M (A) and a mixture of aqueous acetonitrile 50 in 0.1% hydrochloric acid 5 M (B) and

were pumped through the column at 0.7 ml/min. The following gradient system was used (min/% B): 0/5, 5/5, 40/50, 55/100, 59,9/100, 60/5, with 10 min post-run for both compound and metabolite detections. The eluent was monitored by photodiode array detection at 280 nm and spectra of products obtained over the 200–600 nm range. Peaks were characterised by their retention time and spectra characteristics. A calibration curve of phloroglucinol was constructed using authentic standard (0.1–100 µg/ml) and in each case was found to be linear with correlation coefficients of 0.995. Metabolites were quantified as phloroglucinol equivalents.

HPLC-DAD analysis of the urine (Supplemental Table 2) and plasma (Supplemental Table 3) samples, with and without glucuronidase/sulfatase treatment, showed the presence of a variety of metabolites absent in the baselines (before the intervention) in both plasma and urine samples. Some metabolite peaks were present in samples with and without enzymatic treatment, and therefore could be assigned to un-conjugated metabolites. Some other metabolite peaks were present only in samples without enzymatic treatment or were only appearing in samples enzymatically treated, and were attributed to conjugated forms (glucuronides and/or sulfates) and their enzymatically released un-conjugated forms. There was substantial inter-individual variation in (poly)phenol s excreted by the participants. In 42 participants, no (poly)phenol s were detected in urine and in the remainder (n=36) the amounts excreted ranged from 0.5 to 11.8 mg/day (mean  $8.2 \pm 12.1$  mg/d). Similarly, there was a large variation in plasma concentrations ( $42.1 \pm 117.0$  µg/ml),

with only 14 participants having detectable (poly)phenol s in their plasma, seven of which had no (poly)phenol s detected in their urine samples, in accordance with what was observed after acute consumption. It is worth clarifying that, despite inter-individual variability in polyphenols metabolism being common for many polyphenol classes, in the present case the observed variability could partially be the result of the methodological limitations with the HPLC-DAD analysis.

### Supplemental Table 1

RP-HPLC analysis of seaweed metabolites in urine samples. Metabolites were detected in 36 out of 78 participants.

RT (min)	Metabolite	Mean (mg)	Stdev	Volunteers (n)	Enz (-)	Enz (+)
11.9	UM1	11.82	14.14	4	yes	yes
12.8	UM2	3.63	3.03	2	yes	yes
13.1	UM3	6.87	5.01	6	yes	yes
16.3	UM4	3.31	0.03	2	yes	yes
18.6	UM5	3.66	2.07	4	yes	no
21.7	UM6	6.17	3.16	4	yes	yes
24.5	UM7	3.46	2.02	4	no	yes

Online Supporting Material

25.2	UM8	0.47	0.26	2	yes	no
25.9	UM9	2.26	1.18	3	yes	no
28.8	UM10	0.79	0.53	2	no	yes
35.0	UM11	1.57	0.95	3	yes	yes
36.4	UM12	1.02	0.49	4	yes	yes
39.5	UM13	1.18	1.02	4	yes	yes
41.5	UM14	0.95	0.92	4	no	yes
44.2	UM15	0.92	0.74	6	no	yes
48.0	UM16	2.38	2.14	4	yes	no
49.6	UM17	5.42	5.23	5	no	yes
53.8	UM18	0.90	1.17	3	no	yes
<b>Group Mean Total</b>		8.16	12.10	36		

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Phenolics in urine were quantified as phloroglucinol equivalents using HPLC and expressed as mg/d in urine. Note; the row “Group Mean Total ” represents the average of the sum (total excretion) of metabolites calculated for each individual volunteer.

**Supplemental Table 2**

RP-HPLC analysis of seaweed metabolites in plasma samples. Metabolites were detected in 14 out of 78 participants.

RT (min)	Metabolite	Mean ( $\mu\text{g/ml}$ )	Stdev	Volunteers (n)	Enz (-)	Enz (+)
8.5	PM1	148.12	91.12	2	no	yes
9.1	PM2	0.75	0.31	2	yes	no
11.0	PM3	23.71	12.69	2	yes	no
14.9	PM4	110.85	139.63	2	yes	no
31.0	PM5	2.56	1.54	3	yes	no
37.3	PM6	1.55	0.69	2	yes	yes
43.2	PM7	1.34	0.21	2	no	yes
45.3	PM8	0.64	0.18	2	no	yes
48.0	PM9	0.78	0.16	3	yes	no
58.7	PM10	2.62	0.56	2	yes	yes
<b>Group Mean Total</b>		42.08	117.06	14		

Phenolics in plasma were quantified as phloroglucinol equivalents using HPLC and expressed as  $\mu\text{g/ml}$  in plasma.

## Online Supporting Material

Note; the row “Group Mean Total ” represents the average of the sum (total excretion) of metabolites calculated for each individual volunteer.

**Supplemental Table 3.** List of urine metabolites correlated with the chronic intake of seaweed capsules in group 1 formed by 70 participants.

Experimental			Molecular
ID	VIP value	Weight	Metabolite tentative identification
41	5.8	332.18323	Unknown
67	6.0	332.18323	Unknown
165	5.2	182.99788	Saccharin
292	5.0	182.99797	Saccharin
298	5.1	176.06742	3-hydroxy-2-methoxyglutarate/glycerol-1,2-diacetate/3-isopropylmalate/2,3-dimethyl-3-hydroxyglutaric acid/2-isopropylmalic acid
328	5.6	693.32784	Unknown
372	5.1	182.9979	Saccharin
416	4.9	182.99769	Saccharin
457	4.6	234.01896	Ajoene/4-(2,2'-bithiophen-5-yl)-3-butyne-1-ol
464	5.8	183.0885	Epinephrine/tetrahydrohippuric acid/normetanephrine
500	5.4	188.10399	Azelaic acid/butyl ethyl malonate/entre otros
505	4.9	188.10391	Azelaic acid/butyl ethyl malonate/entre otros
556	5.3	183.08848	Epinephrine/tetrahydrohippuric acid/normetanephrine
557	4.5	188.10397	Azelaic acid/butyl ethyl malonate/entre otros
602	4.5	174.9926	Unknown
650	6.6	382.16221	Ibuprofen glucuronide/cyclocalopin B
652	3.8	205.98811	Pyrogallol-O-sulfate
800	4.2	205.98816	Pyrogallol-O-sulfate
801	5.2	182.99757	Saccharin
847	3.3	350.1569	Eremopetasin sulfoxide
940	4.2	692.32582	Pisumoside B
956	5.3	372.10485	Dihydroferulic acid-4-O-glucuronide
981	3.6	440.16758	Unknown



# Online Supporting Material

1025	4.0	351.09499	Indole-3-acetic-acid-O-glucuronide
1106	5.8	150.01492	Tartalic acid/2,3-dihydroxybutanedioic acid/2-2-trienylfuran
1130	3.7	440.16786	Unknown
1155	3.9	440.16724	Unknown
1213	5.0	542.27235	Cortolone-3-glucuronide
1217	4.3	134.05685	2,3-dihydroxyvaleric acid/deoxyribose/dihydroxy-isovalerate/glycerol acetate
1248	5.2	150.0146	Tartalic acid/2,3-dihydroxybutanedioic acid/2-2-trienylfuran
1262	4.4	134.05696	2,3-dihydroxyvaleric acid/deoxyribose/dihydroxy-isovalerate/glycerol acetate
1269	4.6	134.05687	2,3-dihydroxyvaleric acid/deoxyribose/dihydroxy-isovalerate/glycerol acetate
1303	4.5	134.05689	2,3-dihydroxyvaleric acid/deoxyribose/dihydroxy-isovalerate/glycerol acetate
1306	4.2	440.16771	Unknown
1315	6.1	150.01506	Tartalic acid/2,3-dihydroxybutanedioic acid/2-2-trienylfuran
1352	4.3	486.17277	Hydroxytrifuhaol A-glucuronide
1408	4.3	810.29457	Unknown
1429	4.5	356.11012	Hydroxy-methoxycinnamoyl-b-glucose/1-O-feruloylglucose/veranisatin B
1453	3.8	486.17314	Hydroxytrifuhaol A-glucuronide
1458	4.2	486.1734	Hydroxytrifuhaol A-glucuronide
1472	3.9	205.98794	Pyrogallol-O-sulfate
1483	4.4	486.17327	Hydroxytrifuhaol A-glucuronide
1493	5.0	310.97636	Unknown
1544	4.7	824.274	Unknown
1648	5.3	233.03502	Dopamine sulfate
1665	4.8	435.18873	Unknown
1679	4.7	219.08849	N-deschlorobenzoyl indomethacin/alpha-methoxy-1-H-indole-3-propanoic acid/nigellimine N-oxide/alpha-hydroxy-1H-indole-3-propanoic acid
1693	5.4	251.0786	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspatic acid/4-hydroxy-3-methoxy-cinnamoylglycine
1711	4.8	233.0349	Dopamine sulfate
1715	4.6	181.07286	Tyrosine
1725	4.2	298.10513	2-Phenylethanol glucuronide

# Online Supporting Material

1727	5.2	251.07833	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspatic acid/4-hydroxy-3-methoxy-cinnamoylglycine
1737	5.0	219.0888	N-deschlorobenzoyl indomethacin/alpha-methoxy-1-H-indole-3-propanoic acid/nigellimine N-oxide/alpha-hydroxy-1H-indole-3-propanoic acid
1751	5.3	414.15204	Ptelatoside A
1778	5.1	251.0785	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspatic acid/4-hydroxy-3-methoxy-cinnamoylglycine
1799	5.4	842.39386	Unknown
1806	3.5	338.947	Unknown
1828	6.1	364.17303	Unknown
1877	5.2	842.39393	Unknown
1917	5.0	544.28816	Dioxinodehydroeckol glucuronide
1924	4.7	360.1411	Dimethoxyphenyl-ethanediol O-glucoside/2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol 4'-glucoside/ deoxyloganic acid
1935	4.7	223.11987	Cerulenin
1945	4.9	251.0785	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspatic acid/4-hydroxy-3-methoxy-cinnamoylglycine
1948	4.2	218.02395	tyrosol-sulfate/diphenyl disulfide
1953	4.1	378.05751	Unknown
1961	4.9	400.1728	Corchoionoside B
1970	4.7	223.11986	Cerulenin
1995	5.1	372.10472	Dihydroferulic acid-4-O-glucuronide
2038	5.3	360.14121	Dimethoxyphenyl-ethanediol O-glucoside/2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol 4'-glucoside/ deoxyloganic acid
2040	5.5	526.27719	Unknown
2059	5.4	504.19905	Myricatomentoside I/chlorhexidine
2063	4.4	398.10594	Methyl 3,4-dihydroxy-5-prenylbenzoate-3-glucoside
2072	4.4	372.10487	Dihydroferulic acid-4-O-glucuronide
2080	5.4	324.06294	Sterigmatocystin/greviline A

## Online Supporting Material

2081	4.6	251.07794	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspatic acid/4-hydroxy-3-methoxy-cinnamoylglycine
2185	4.8	280.05956	Unknown
2186	4.8	280.05942	Unknown
2188	4.8	240.12161	Anserine/homocarnosine/balenine
2194	5.4	213.00886	Indoxyl sulfate/carmustine

All predicted formula derived with < 5 ppm mass accuracy data. VIP (variable influence in projection) is a variable that summarizes the importance of X variables to the OPLS-DA model. Variables with VIP values > 2 were the main contributors in the model. ID, identification number. Metabolites were searched against two data bases, (Metlin database; <http://metlin.scripps.edu/> and HMDB: <http://hmdb.ca>). Searching metabolites do not include heavy metal and halogens.

**Supplemental Table 4.** List of plasma metabolites correlated with the chronic intake of seaweed capsules.

<b>ID</b>	<b>VIP value</b>	<b>Experimental weight</b>	<b>Molecular</b>	<b>Tentative identification</b>
1315	2.1	150.01506		Tartalic acid/2,3-dihydroxybutanedioic acid/2-2-trienylfuran
1450	3.1	188.01341		p-cresol sulfate
246	2.2	195.95296		Unknown
114	2.4	195.953		Unknown
111	2.3	195.95304		Unknown
977	2.3	208.00368		Unknown
2066	2.1	210.03589		Galactaric acid/glucaric acid
797	2.0	213.00848		Indoxyl sulfate/carmustine
1948	2.0	218.02395		tyrosol-sulfate/diphenyl disulfide
1711	2.1	233.0349		Dopamine sulfate
1648	2.3	233.03502		Dopamine sulfate
2097	2.2	233.03511		Dopamine sulfate
587	2.0	236.10413		4-hydroxy-3-methoxy-5-(3-methylbut-2-en-1-yl)benzoic acid/carboxy-ibuprofen/ethyl vanillin isobutyrate
496	2.0	236.10422		4-hydroxy-3-methoxy-5-(3-methylbut-2-en-1-yl)benzoic acid/carboxy-ibuprofen/ethyl vanillin isobutyrate
892	2.1	246.0189		Unknown
2138	2.7	278.03987		Unknown
2186	3.2	280.05942		Unknown
2185	3.3	280.05956		Unknown
706	2.5	293.9301		Unknown
622	2.1	293.9301		Unknown
2119	3.2	324.12077		Unknown
2035	3.0	324.12114		Unknown
588	2.1	326.13624		4-(4'-hydroxyphenyl)2-butanone glucoside/ citrusin C/1-methoxy-3-(4-hydroxyphenyl)-2E-propenal 4'-glucoside

# Online Supporting Material

654	2.4	326.13646	4-(4'-hydroxyphenyl)2-butanone glucoside/ citrusin C/1-methoxy-3-(4-hydroxyphenyl)-2E-propenal 4'-glucoside
1293	2.3	330.16753	Diphlorethol sulfate
1356	2.3	330.16795	Diphlorethol sulfate
2163	2.0	336.0294	Unknown
2013	2.0	336.02953	Unknown
1958	2.2	347.14012	Unknown
2068	2.5	358.08919	Dihydrocaffeic acid -O-glucuronide
519	2.6	360.14168	Dimethoxyphenyl-ethanediol O-glucoside/2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol 4'-glucoside/ deoxyloganic acid
430	2.2	360.14198	Dimethoxyphenyl-ethanediol O-glucoside/2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol 4'-glucoside/ deoxyloganic acid
1540	2.3	380.14656	Gibberellin A75/8-oxodiacetoxyscirpenol
1357	2.0	382.16224	Ibuprofen glucuronide/cyclocalopin B
381	2.4	398.15706	Methyl 3,4-dihydroxy-5-prenylbenzoate-3-glucoside
364	2.0	398.15764	Methyl 3,4-dihydroxy-5-prenylbenzoate-3-glucoside
234	2.0	412.13594	Methylpicraquassioside A
134	2.3	412.136	Methylpicraquassioside A
214	2.0	412.13618	Methylpicraquassioside A
154	2.0	412.13626	Methylpicraquassioside A
159	2.2	412.13637	Methylpicraquassioside A
164	2.2	412.13649	Methylpicraquassioside A
1751	2.0	414.15204	Ptelatoside A
2134	2.1	440.09245	3,5-dihydroxyphenyl-1-O-(6-O-galloyl-beta-D-glucopyranoside)
1633	2.1	544.21522	Dioxinodehydroeckol glucuronide
351	2.0	796.31532	Unknown
853	2.1	797.31862	Fucophloroethols glucuronide
1182	2.0	169.0363	2-furoylglycine/L-2,3-dihydrodipicolinate
1627	2.4	174.08776	Suberic acid/ 2-propylglutaric acid
753	2.1	174.99243	Unknown

# Online Supporting Material

1083	2.1	174.99252	Unknown
602	2.8	174.9926	Unknown
859	2.3	174.9926	Unknown
1023	2.1	174.9926	Unknown
769	2.0	174.99263	Unknown
2094	2.3	189.00848	Lanthionine ketimine/2-aminophenol sulphate
244	2.1	229.00336	Unknown
702	2.0	248.0315	C-O-C dimer of phloroglucinol
1918	2.8	259.99821	Caffeic acid 3-sulfate
887	2.2	261.03016	Unknown
1120	2.8	290.04494	4-hydroxy-5-(hydroxyphenyl)-valeric acid-sulfate
1677	2.1	303.05847	Unknown
1481	2.9	314.09898	2-hydroxyphenylacetic acid-O-glucuronide
1993	2.1	314.09898	2-hydroxyphenylacetic acid-O-glucuronide
293	2.1	327.09512	C-O-C dimer of phloroglucinol-sulfate
839	2.1	328.09828	Difucol/diphlorethol glucuronide
952	2.2	350.02393	Unknown
847	2.0	350.1569	Eremopetasin sulfoxide
1066	2.4	357.10545	HMBOA-GLc (known as 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one
625	2.2	357.10548	HMBOA-GLc (known as 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one
479	2.1	357.1056	HMBOA-GLc (known as 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one
626	2.4	357.10573	HMBOA-GLc (known as 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one
1966	2.0	358.08912	Dihydrocaffeic acid-O-glucuronide
1620	2.1	359.06694	Unknown
1351	2.3	362.15735	Unknown
870	2.4	362.15748	Unknown
1792	2.9	368.10989	3-O-feruloylquinic acid/O-caffeoyl-O-methylquinic acid

# Online Supporting Material

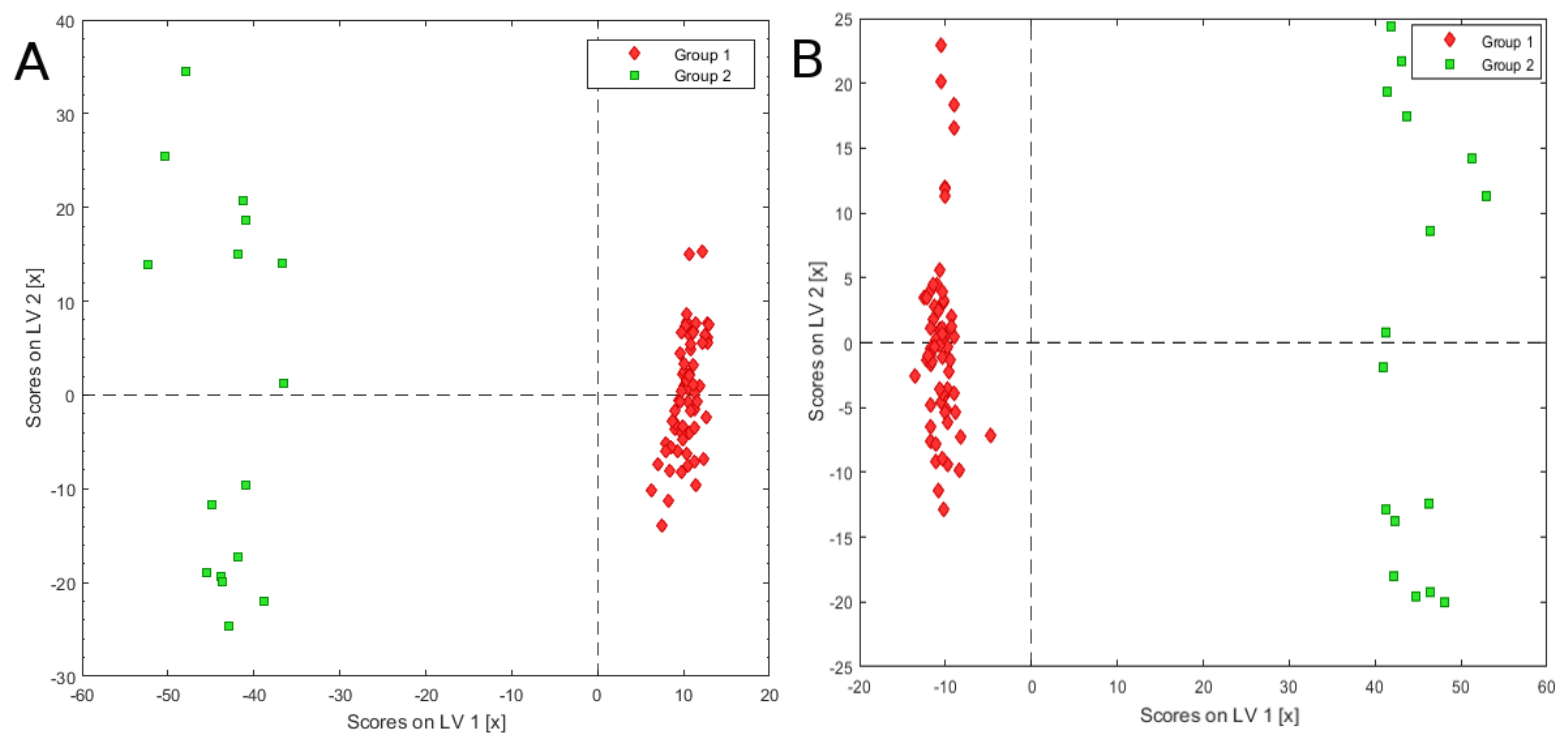
1758	2.4	370.08925	Feruloyl C-glucuronide/isoferulic acid-3-O-glucuronide/5-hydroxy-6-methoxycoumarin7-glucoside	
1683	2.0	370.08926	Feruloyl C-glucuronide/isoferulic acid-3-O-glucuronide/5-hydroxy-6-methoxycoumarin7-glucoside	
1788	2.3	370.08955	Feruloyl C-glucuronide/isoferulic acid-3-O-glucuronide/5-hydroxy-6-methoxycoumarin7-glucoside	
1682	2.9	372.10469	Dihydroferulic acid-4-O-glucuronide	
1757	2.8	372.10469	Dihydroferulic acid-4-O-glucuronide/veranisatin C	
1634	2.6	372.10469	Dihydroferulic acid-4-O-glucuronide	
1765	2.9	372.10475	Dihydroferulic acid-4-O-glucuronide	
1719	2.9	372.10491	Dihydroferulic acid-4-O-glucuronide	
1570	2.1	372.10499	Veranisatin C/dihydroferulic acid-glucuronide	
1776	2.2	380.16774	3-Methyl-3-butenyl apiosyl-glucoside/prenyl arabinosyl-glucoside/ecabet	
1496	2.8	384.05021	Unknown	
2017	3.0	384.12615	Unknown	
1512	2.0	388.17252	hydroxypropyl-2-methoxyphenoxyl-1,3-propanediol xyloside/jasmonic acid derivative	1
1941	2.1	388.17283	hydroxypropyl-2-methoxyphenoxyl-1,3-propanediol xyloside/jasmonic acid derivative	1
1638	2.7	388.17311	hydroxypropyl-2-methoxyphenoxyl-1,3-propanediol xyloside/jasmonic acid derivative	1
1330	2.7	410.04463	Unknown	
1768	2.8	410.0451	Unknown	
1341	2.4	446.09862	Unknown	
1551	2.3	446.09904	Unknown	
1664	2.0	496.23067	Glaucarubin	
1781	2.5	509.13896	trans-hydroxycyclohexyl glyburide (Benzenesulfonamides)	

All predicted formula derived with < 5 ppm mass accuracy data. VIP (variable influence in projection) is a variable that summarizes the importance of X variables to the OPLS-DA model. Variables with VIP values > 2 were the main

## Online Supporting Material

contributors in the model. ID, identification number. Metabolites were searched against two data bases, (Metlin database; <http://metlin.scripps.edu/> and HMDB: <http://hmdb.ca>). Searching metabolites do not include heavy metal and halogens.



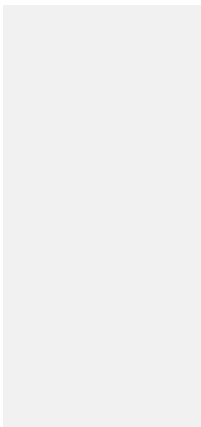


**Supplemental Figure 3.** OPLS-DA scores plots of the urine samples belong to group of participants 1 (70 volunteers-red) and group of subject 2 (8 participants-green) before (A) and after (B) seaweed consumption. LV1: latent variable 1; LV 2: latent variable 2.

## Supplemental table 5 Total ex

Subject Id	Pyrogallol/Phloroglucinol-sulfate
S58	0.000
S61	0.000
S76	0.000
S78	0.000
S60	0.000
S70	0.003
S79	0.000
S83	0.000
S64	0.015
S40	0.047
S27	0.057
S66	0.064
S25	0.040
S65	0.085
S30	0.040
S71	0.000
S10	0.070
S33	0.128
S03	0.085
S59	0.111
S74	0.098
S19	0.156
S43	0.176
S39	0.085
S75	0.065
S01	0.184
S15	0.130
S53	0.125
S41	0.209
S36	0.225
S49	0.327
S82	0.328
S04	0.312
S46	0.347
S57	0.407
S81	0.373
S54	0.232
S35	0.204
S67	0.235
S16	0.447
S05	0.060
S23	0.547
S17	0.508
S08	0.302
S52	0.477
S29	0.407
S56	0.513

S02	0.532
S18	0.635
S38	0.337
S34	0.377
S31	0.531
S21	0.417
S24	0.453
S55	0.596
S22	0.500
S11	0.514
S37	0.287
S48	0.604
S42	0.436
S44	0.846
S63	0.535
S06	0.601
S12	0.366
S13	0.317
S62	0.555
S14	0.640
S20	0.843
S72	0.772
S32	0.682
S73	0.711
S26	0.533
S77	1.089
S07	0.622
S09	0.867
S51	1.158
S47	0.731
S50	1.470



## Excretion of identified seaweed polyphenols in urine (mMol)

Pyrogallol/Phloroglucinol-sulfate	Pyrogallol/Phloroglucinol-sulfate
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.004	0.004
0.000	0.000
0.000	0.000
0.016	0.022
0.036	0.004
0.064	0.012
0.091	0.012
0.026	0.019
0.055	0.110
0.062	0.007
0.000	0.000
0.078	0.020
0.259	0.018
0.095	0.017
0.214	0.015
0.148	0.003
0.114	0.026
0.285	0.030
0.119	0.011
0.263	0.023
0.171	0.106
0.105	0.035
0.081	0.093
0.319	0.034
0.313	0.021
0.327	0.090
0.212	0.037
0.348	0.048
0.417	0.014
0.514	0.014
0.570	0.046
0.583	0.045
0.253	0.037
0.493	0.063
0.616	0.074
0.052	0.045
0.320	0.062
0.574	0.108
0.373	0.046
0.511	0.083
0.586	0.056
0.473	0.193

0.662	0.055
0.543	0.074
0.261	0.716
0.580	0.069
0.867	0.085
0.334	0.183
0.573	0.062
0.671	0.094
0.720	0.083
0.693	0.043
0.628	0.058
0.714	0.081
0.340	0.591
0.611	0.157
0.952	0.067
0.673	0.111
0.452	0.042
0.246	0.402
0.944	0.077
0.964	0.037
0.786	0.318
1.026	0.031
1.178	0.105
1.223	0.120
1.039	0.129
0.898	0.284
0.884	0.072
0.977	0.126
0.827	0.167
1.534	0.132
0.984	0.216

oles)	
Participants	
Hydroxytrifuhaol-A-glucuronide	
	0.000
	0.000
	0.000
	0.000
	0.000
	0.000
	0.000
	0.000
	0.000
	0.029
	0.034
	0.023
	0.062
	0.017
	0.068
	0.000
	0.090
	0.019
	0.094
	0.063
	0.076
	0.073
	0.031
	0.117
	0.093
	0.069
	0.146
	0.156
	0.105
	0.105
	0.076
	0.095
	0.112
	0.095
	0.074
	0.062
	0.123
	0.206
	0.149
	0.064
	0.331
	0.127
	0.070
	0.217
	0.142
	0.120
	0.060

0.110  
0.065  
0.042  
0.195  
0.041  
0.202  
0.171  
0.103  
0.142  
0.188  
0.216  
0.120  
0.130  
0.089  
0.120  
0.180  
0.375  
0.382  
0.162  
0.165  
0.081  
0.127  
0.086  
0.086  
0.287  
0.078  
0.323  
0.249  
0.181  
0.117  
0.378

Total mmoles excreted in urine		
Hydroxytrifuhaol-A-glucuronide	Hydroxytrifuhaol-A-glucuronide	
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.027	0.028
	0.030	0.031
	0.025	0.028
	0.055	0.039
	0.022	0.025
	0.066	0.064
	0.000	0.000
	0.080	0.082
	0.016	0.015
	0.083	0.084
	0.050	0.043
	0.069	0.069
	0.061	0.061
	0.032	0.032
	0.113	0.106
	0.082	0.085
	0.063	0.063
	0.133	0.133
	0.133	0.142
	0.088	0.087
	0.091	0.086
	0.068	0.069
	0.097	0.097
	0.095	0.101
	0.084	0.083
	0.070	0.067
	0.054	0.056
	0.121	0.109
	0.199	0.187
	0.141	0.135
	0.062	0.058
	0.299	0.301
	0.151	0.115
	0.066	0.064
	0.193	0.193
	0.117	0.102
	0.129	0.129
	0.115	0.134



0.097	0.093
0.104	0.107
0.081	0.103
0.167	0.153
0.045	0.048
0.193	0.185
0.166	0.155
0.102	0.094
0.126	0.129
0.177	0.165
0.242	0.196
0.117	0.109
0.134	0.126
0.080	0.081
0.098	0.109
0.159	0.163
0.318	0.341
0.335	0.347
0.172	0.147
0.147	0.150
0.076	0.074
0.122	0.116
0.096	0.102
0.080	0.078
0.250	0.229
0.089	0.088
0.286	0.293
0.221	0.226
0.164	0.164
0.108	0.107
0.411	0.343

(N=78)		
Hydroxytrifuhaol-A-glucuronide	Dioxinodehydroeckol-glucuronide	
	0.000	0.000
	0.000	0.000
	0.000	0.001
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.001
	0.029	0.000
	0.029	0.000
	0.028	0.000
	0.035	0.001
	0.026	0.000
	0.059	0.000
	0.000	0.000
	0.037	0.000
	0.017	0.000
	0.038	0.001
	0.028	0.000
	0.066	0.000
	0.069	0.000
	0.032	0.000
	0.099	0.001
	0.071	0.000
	0.057	0.000
	0.128	0.001
	0.143	0.000
	0.056	0.001
	0.060	0.000
	0.006	0.000
	0.105	0.000
	0.090	0.000
	0.066	0.000
	0.065	0.000
	0.055	0.001
	0.013	0.000
	0.167	0.000
	0.121	0.000
	0.059	0.000
	0.304	0.000
	0.107	0.001
	0.059	0.000
	0.151	0.000
	0.050	0.000
	0.122	0.000
	0.127	0.000

0.082	0.000
0.113	0.001
0.116	0.000
0.115	0.001
0.047	0.000
0.187	0.001
0.151	0.001
0.094	0.001
0.095	0.000
0.018	0.000
0.194	0.000
0.107	0.000
0.125	0.001
0.076	0.000
0.105	0.000
0.120	0.001
0.182	0.000
0.160	0.002
0.149	0.000
0.116	0.000
0.070	0.000
0.110	0.000
0.108	0.001
0.079	0.000
0.132	0.001
0.089	0.001
0.217	0.001
0.103	0.000
0.162	0.000
0.102	0.001
0.338	0.000

[illegible]

[illegible]

[illegible]

[illegible]

Diphlorethol sulfate	Total metabolites (mmoles)
0.000	0.001
0.000	0.001
0.001	0.003
0.001	0.003
0.002	0.006
0.000	0.012
0.008	0.019
0.001	0.047
0.000	0.055
0.000	0.200
0.000	0.258
0.000	0.270
0.000	0.278
0.000	0.341
0.000	0.367
0.004	0.405
0.000	0.459
0.000	0.473
0.000	0.498
0.000	0.525
0.000	0.530
0.000	0.561
0.000	0.618
0.000	0.651
0.000	0.683
0.000	0.714
0.000	0.812
0.000	0.874
0.000	0.899
0.000	0.902
0.000	0.964
0.000	0.972
0.000	1.106
0.000	1.107
0.000	1.212
0.000	1.216
0.000	1.227
0.000	1.254
0.000	1.338
0.000	1.381
0.000	1.394
0.000	1.430
0.000	1.450
0.000	1.477
0.000	1.483
0.000	1.550
0.000	1.616



0.000	1.632
0.000	1.642
0.000	1.657
0.000	1.660
0.000	1.665
0.000	1.701
0.000	1.733
0.000	1.756
0.000	1.796
0.000	1.797
0.000	1.821
0.000	1.852
0.000	1.883
0.000	1.942
0.000	1.986
0.000	2.007
0.000	2.078
0.000	2.192
0.000	2.206
0.000	2.220
0.000	2.249
0.000	2.305
0.000	2.357
0.000	2.377
0.000	2.600
0.000	2.617
0.000	2.699
0.000	2.769
0.000	2.825
0.000	2.832
0.000	4.140

Mean	1.29
Sd	0.88
min	0.001
max	4.140

[illegible]

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1

Low (<.5 mmole	High>2mmole
19	16

Subject Id	Total meta	Low (<.5 mmole =1	High>2mmole =2
S58	0.00	1.00	
S61	0.00	1.00	
S76	0.00	1.00	
S78	0.00	1.00	
S60	0.01	1.00	
S70	0.01	1.00	
S79	0.02	1.00	
S83	0.05	1.00	
S64	0.05	1.00	
S40	0.20	1.00	
S27	0.26	1.00	
S66	0.27	1.00	
S25	0.28	1.00	
S65	0.34	1.00	
S30	0.37	1.00	
S71	0.40	1.00	
S10	0.46	1.00	
S33	0.47	1.00	
S03	0.50	1.00	
S59	0.53		
S74	0.53		
S19	0.56		
S43	0.62		
S39	0.65		
S75	0.68		
S01	0.71		
S15	0.81		
S53	0.87		
S41	0.90		
S36	0.90		
S49	0.96		
S82	0.97		
S04	1.11		
S46	1.11		
S57	1.21		
S81	1.22		
S54	1.23		
S35	1.25		
S67	1.34		
S16	1.38		
S05	1.39		
S23	1.43		
S17	1.45		
S08	1.48		
S52	1.48		
S29	1.55		
S56	1.62		
S02	1.63		
S18	1.64		

S38	1.66	
S34	1.66	
S31	1.67	
S21	1.70	
S24	1.73	
S55	1.76	
S22	1.80	
S11	1.80	
S37	1.82	
S48	1.85	
S42	1.88	
S44	1.94	
S63	1.99	
S06	2.01	2.00
S12	2.08	2.00
S13	2.19	2.00
S62	2.21	2.00
S14	2.22	2.00
S20	2.25	2.00
S72	2.31	2.00
S32	2.36	2.00
S73	2.38	2.00
S26	2.60	2.00
S77	2.62	2.00
S07	2.70	2.00
S09	2.77	2.00
S51	2.82	2.00
S47	2.83	2.00
S50	4.14	2.00